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Effects of Neonicotinoid Pesticides on Bumblebee Social Behaviour

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Abstract

Social bees are the single most important group of pollinators, yet many populations are in decline and human activity is the likely cause. In agricultural landscapes, pollinating bees are unintentionally exposed to a diverse cocktail of chemical pesticides; such exposure is thought to be a significant driver in the decline of bee populations worldwide. Systemic neonicotinoid pesticides are of particular concern because they are widely used and they have a direct route of exposure to bees via the nectar and pollen of treated crops. Field-realistic sub-lethal doses of these insecticides disrupt nerve cells in the bee brain, leading to effects on individual behaviour and colony productivity. These effects are well described, but we do not understand the mechanisms by which effects on individuals scale up to colony-level failure. This thesis shows that social context modulates neonicotinoid-induced behavioural impairments in bumblebees (*Bombus terrestris*) and that colonies exhibit a certain level of resilience that could not have been predicted based on individual responses alone. High-throughput automated behavioural monitoring revealed that the movement speeds of bees decreased, but bees tended to cluster together to maintain social interaction rates. The nest behaviour of active foragers (relative to non-foraging workers) showed the greatest susceptibility to toxic effects and did not recover post-exposure. However, total colony-level foraging effort remained relatively unchanged. Foragers also showed normal interactions patterns with non-foragers and temporal network flow simulations suggested this would maintain information flow across the colony. Additionally, behaviourally dominant bees seemed to be relatively more strongly affected, but again, group-level social organisation (dominance hierarchy formation) was not affected. These results demonstrate the importance of assessing the risks of pesticide exposure to bumblebees in a social context. Furthermore, the emergence of social organisation through the self-organising patterns of pair-wise interactions may be a key mechanism providing social resilience to pesticides. Understanding the responses of complex social systems, as found in insect societies, is vital to predicting how insects will cope with increasing agricultural intensification.

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Finally, I would like to dedicate this work to my parents. They knew I would be a biologist since I was five years old and they have helped me get here ever since. I couldn't have made it without you and your help on all those school science projects.

Contributors

Chapter 3 and Chapter 4

Experiments designed and carried out by primarily by SD. Josh Gabbatiss and Claire Narraway provided assistance in marking bees and recording colony videos.

Chapter 5

Experiment designed by SD. Experiments conducted by Freddie Wilkinson and Cecylia Watrobska. Behavioural observations carried out by Fenn Cullen and verified by SD.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE:.....

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"Everybody knows the burly, good-natured bumble-bee. Clothed in her lovely coat of fur, she is the life of the gay garden as well as of the modestly blooming wayside as she eagerly hums from flower to flower, diligently collecting nectar and pollen from the break to the close of day."

F. W. L. Sladen (1912) *The Humble-Bee*

Chapter 1

Introduction

Human activity is affecting the Earth in such profound ways that the actions of our species could define a distinct geological epoch: the Anthropocene (Waters et al., 2016). One of the markers of this new era is the ongoing and accelerating loss of biodiversity, which has been referred to as "the sixth mass extinction" (Barnosky et al., 2011; Ceballos et al., 2017). The loss of species (and their interactions) has the potential to disrupt the functioning of entire ecosystems, and thus the benefits that humans derive from them (Cardinale et al., 2012). The majority of these benefits, often referred to as ecosystem services, are thought to be in a state of degradation or are unsustainably managed (Millenium Ecosystems Assessment, 2005). In recent decades, however, global food production has risen in line with the demands of the increasing human population (7.6 billion people alive today) and is expected to have to double by 2050 to keep up [9.8 billion people projected to be living in 2050 (United Nations et al., 2017)]. Yet, cropland is limited and converting natural habitats to agricultural has already degraded ecosystems worldwide (Zhang et al., 2006). Therefore, to ensure food security in the future it is necessary to increase the productivity of current agricultural land and prevent further environmental harm.

The contemporary global food production has managed to continue to increase production thanks to the technological advances and agricultural practices developed during the Green Revolution; of which, the use of pesticides has played a central role (Pingali, 2012). Around 40% of global crop production is destroyed by pests, but without the $40 billion invested in pesticides losses could run as high as 70% (Pimentel, 2009). Despite these benefits to crop protection, there is growing concern that the 3 million metric tonnes of chemical pesticides applied across the world annually are seriously impacting the health of natural plants, animals and ecosystems (Pimentel, 2009). The impacts of pesticides on pollinators are of particular concern. Pollinators provide a vital ecosystem service by pollinating many crops, but in doing so they are directly exposed to pesticides which harm their health and thought to be a key driver of pollinator population declines (Vanbergen and Insect Pollinators Initiative, 2013). Ultimately, the environmental costs of agricultural pesticides are predicted to restrict the growth of food production in the future (Godfray et al., 2010). This trade-off between chemical crop protection and the conservation of ecosystem services puts global food security at risk, which leads to the question: how do we provide reliable access to sufficient and nutritious food for the world's population in a sustainable way? The answer is, of course, complex, but this thesis aims to contribute to our assessment of this trade-off by further understanding the level of risk that pesticides pose to social insect pollinators.

1.1 The Pollinator Crisis

Pollinators facilitate and enhance reproduction of the vast majority of flowering plants and thus play a vital role in maintaining the biodiversity and ecosystem services of terrestrial ecosystems worldwide (Ollerton et al., 2011).

Humans are also directly reliant on biotic pollination for 35% of global food production (Klein et al., 2007), which includes many nutrient-rich fruits, vegetables, nuts and seeds essential to human health and wellbeing (Eilers et al., 2011). Globally, both the yield and acreage of pollinator-dependent crops have been increasing over recent decades and production is currently meeting demand (Aizen et al., 2008; Breeze et al., 2011). The vast majority of pollination services are provided by insects, including diverse wild populations and a handful of managed species (Orford et al., 2015; Rader et al., 2016). Furthermore, the presence of a high diversity and abundance of insect pollinators has been shown to increase the yield and improve the quality of many crops types (Bommarco et al., 2012; Garibaldi et al., 2013). However, mounting evidence suggests that both wild and managed pollinators are experiencing significant and rapid declines (Biesmeijer et al., 2006; Cameron et al., 2011; Ellis et al., 2010; Potts et al., 2010a; Potts et al., 2010b). These two divergent trends, increasing dependence on animal-pollinated crops and declining pollinator numbers, have led to grave concerns that an impending 'pollinator crisis' could add yet more strain on global food security (Aizen and Harder, 2009; Breeze et al., 2014; Gallai et al., 2009; Garibaldi et al., 2011; Teichroew et al., 2017). If we are to avoid a sudden collapse in pollination services (Lever et al., 2014) we must understand the complex causes and consequences of pollinator declines.

There is no single cause of pollinator declines; on the contrary, many interacting human-mediated pressures are thought to be afflicting pollinator health, abundance and diversity (Vanbergen and Insect Pollinators Initiative, 2013). Ironically, the pressures attributable to agricultural intensification are among the most significant in their impact on pollinators. One example is the loss and fragmentation of natural habitats, which is primarily driven by the

conversion of land to both agriculture and urbanization, and is thought to be a significant contributor to pollinator declines (Biesmeijer et al., 2006). Agricultural land reduces the abundance and diversity of the wild flowering plants that pollinators rely on for food (Biesmeijer et al., 2006). For example, mass-flowering crops such as oil-seed rape can provide an ephemeral surge of abundant forage for pollinators but cannot support populations year-round (Kremen et al., 2007). Land-use intensification can interact with the pressures of climate change; increasingly fragmented habitats can limit the dispersal ability of species faced with unfavourable climatic conditions in their historical range, leading to increased extinction risks (Forister et al., 2010; Kerr et al., 2015). Climate change is also affecting the floral resources available to pollinators via 'phenological mismatch': the asynchrony in the seasonal timing of pollinator activity and flowering periods (Memmott et al., 2007). Plant-pollinator interactions are also affected by the introduction of alien species that can outcompete indigenous species for resources (Dietzsch et al., 2011), with relatively unknown consequences on for native pollination communities. The more insidious, and perhaps more severe, consequence of alien invasions is the co-introduction of novel pests and pathogens. The introduction of non-native bumblebee species for pollination purposes, for example, has caused declines in native pollinators in many regions because the parasites that invade alongside their host spread and infect local species (Graystock et al., 2013; Schmid-Hempel et al., 2014). The non-target exposure of pollinators to broad-spectrum pesticides is another pressure that can interact synergistically with many of those described above (Alaux et al., 2010; Dance et al., 2017). Chemical insecticides designed to kill small herbivorous pests have unintentional routes of exposure to beneficial insect pollinators, which can have serious detrimental effects on pollinator health

(Bonmatin et al., 2014; Pisa et al., 2014; van Lexmond et al., 2014). Pollinators are exposed to a wide variety of pesticides on agricultural land and several landscape-scale studies have shown that areas of high pesticide use are strongly associated with reduced pollinator abundance and diversity (Balfour et al., 2017; Brittain et al., 2010; Woodcock et al., 2016).

Just as there is no single cause of pollinator declines, there is no single answer to conserving these animals and the ecosystem service they provide. Additionally, any efforts to conserve pollinators must balance with efforts to match food demands via agricultural production. The use of pesticides has a critical part to play in this balancing act because of their direct impacts on increased food production and on pollinator health. The development of effective pesticides (including herbicides, fungicides and insecticides) and their widespread use has been one of the single most significant factors in the tremendous increases in crop production seen over the past 70 years (Cooper and Dobson, 2007). Combined pesticide use has enabled farmers to increase the productivity of staple crops such as wheat (Webster et al., 1999), and to greatly improve the commercial viability of producing fruits and vegetables (Cooper and Dobson, 2007). The benefits of appropriate pesticide use to human health and well-being via the provision of adequate and nutritious food cannot be understated. However, widespread prophylactic use of broad-spectrum pesticides has become the norm; this trend goes against the long-established judicious principals of Integrated Pest Management (IPM), does not improve yields, and poses greatly elevated risks of environmental harm (Goulson, 2013; Simon-Delso et al., 2015; van Lexmond et al., 2014). An IPM approach considers all available information to make informed pesticide application decisions, but often farmers do not have a choice: many commercially produced seeds are now pre-treated with systemic insecticides

(the type of pesticides with the highest exposure risk to pollinators) (Simon-Delso et al., 2015). Governments should be taking more of an active role in the regulation of appropriate pesticide use, but such regulatory decisions should be made based on sound scientific evidence. The next section will consider how science and policy have informed the use of neonicotinoids (a specific class of insecticides implicated in pollinator declines) and how a full understanding of the risks of these chemicals has a crucial part to play in achieving a productive agricultural system that does not further exacerbate the plight of pollinators.

1.2 The Rise (and Fall?) of Neonicotinoid Pesticides

Neonicotinoid insecticides were introduced into plant protection products in the 1990s and quickly became the most widely-used class of insecticides in the world (Jeschke et al., 2011), but many of the properties that have made them so popular are also polluting the environment and causing harm to pollinators. Neonicotinoids such as imidacloprid, thiamethoxam, clothianidin and thiacloprid, to name a few, are insect neurotoxins that bind to the nicotinic acetylcholine receptors of the central nervous system (Casida and Durkin, 2013). At low doses, this receptor binding causes nervous stimulation and hyperactivity while higher doses block receptors leading to paralysis and death. This novel mode of action has made neonicotinoids an excellent option for controlling pests that had developed resistance to older pesticides such as organophosphates, pyrethroids, carbamates, and chlorinated hydrocarbons (Nauen and Denholm, 2005). Neonicotinoids also provide high insecticidal potency and specificity (Jeschke and Nauen, 2009), which means they can be effective at relatively low application rates and pose little threat to vertebrates (although other invertebrates seem to be affected, see Pisa et al.,

2014). Additionally, neonicotinoids act as systemic pesticides, meaning they are readily absorbed by plant tissues, making them suitable for a range of application techniques including soil drenching, foliar application and seed treatment (Jeschke and Nauen, 2009). This systemic property is possible because these chemicals are water soluble and relatively stable, which provides effective long-lasting protection against pests (Jeschke and Nauen, 2009). However, this can also lead to wider environmental contamination: neonicotinoids are persistent in treated soil, present in runoff into water systems, and absorbed by nearby wildflowers (Bonmatin et al., 2014; Botías et al., 2016). Widespread neonicotinoid pollution is thought to be causing harm to insects and other arthropods in both aquatic and terrestrial ecosystems (Goulson, 2013; Sánchez-Bayo et al., 2016). Systemic neonicotinoid treatment also poses direct exposure risks to pollinators because the chemicals are translocated throughout the treated plant's tissues, and are thus present in pollen and nectar. In summary, these highly potent insect neurotoxins effectively protect crops all over the world, but also have a direct exposure route to beneficial insect pollinators.

In light of the exposure risk posed by systemic neonicotinoids to pollinators, the use of these insecticides is regulated and they are intended to be applied to crops at a level that is non-lethal to beneficial insects, specifically social bees (for risk assessment protocols in the European Union, see EFSA, 2013; EFSA, 2018b). Measurements of neonicotinoid concentrations in the nectar and pollen of treated crops are typically ≤10 μgkg^{-1} (1 μgkg^{-1} is equivalent to 1 ppb; Blacquière et al., 2012). Nevertheless, many laboratory and field studies have shown that doses of neonicotinoids considered to be field-realistic can have a variety of "sub-lethal" effects on

pollinator health (for concise lists of evidence, see Godfray et al., 2014; Godfray et al., 2015).

Mounting evidence published in scientific literature throughout the 2000s and 2010s suggested that sub-lethal doses of neonicotinoids were contributing to honeybee (*Apis mellifera*) declines in particular (for recent review, see Alkassab and Kirchner, 2016). This wave of neonicotinoid exposure research captured the attention of the public, the press (Walsh, 2013) and policy makers (EFSA, 2012). However, the causal link between neonicotinoid use and changes to social bee populations has not been fully established, which has led to much debate about the claimed role of these pesticides in observed declines. For example, a study by Pilling et al. (2013) was particularly controversial. This multi-year field exposure experiment reported no adverse effects on honeybee colony health and overwintering from foraging on thiamethoxam-treated crops (Pilling et al., 2013). The authors of the study were (or had one been) contracted by the agrochemical company Syngenta to conduct the research, which develops and markets neonicotinoid crop treatments. This competing interest led some to investigate the conclusions of the study more closely. Consequently, Hoppe et al. (2015), a number of scientists from across Europe, challenged the conclusions of Pilling et al. (2013) by highlighting "a number of issues where the experimental approach, methodology, data reporting and analysis, and publication process are unclear, misleading or problematic." Hoppe et al. (2015) concluded "the study is unable to provide any scientific insights into the effects of thiamethoxam on bee colonies in the field". The original authors of the Pilling et al. (2013) study addressed these criticisms, stating that they are "either wrong, inaccurate or misleading." Arguments such as these have led to confusion and

uncertainty around the topic, the answer to which has been calls for more research.

Despite some disputes about the strength of evidence linking neonicotinoid use to effects on bee colony health, the European Commission (EC) determined that neonicotinoids did indeed pose a significant risk to bees (EFSA, 2013). In 2013 the EC took precautionary measures to protect pollinator populations and imposed a temporary moratorium on the use of certain neonicotinoids (clothianidin, thiamethoxam and imidacloprid) on flowering, bee-attractive crops throughout the European Union (EU). Following this precautionary decision in Europe, the European Food Safety Authority (EFSA) announced an open call for more research in 2015 (EFSA, 2015), which was which was eventually published as a review of the evidence in February 2018 (EFSA, 2018b). Ultimately, based on these findings, the EC extended existing restrictions to a permanent ban on all outdoor use of clothianidin, thiamethoxam and imidacloprid from April 2018. The EU ban should provide widespread protection to pollinators in agricultural landscapes across the continent, but the neonicotinoid story is not over. Neonicotinoid residues have been detected in UK honey since the initial EU moratorium, suggesting persistent soil contamination could continue to pose an exposure risk to pollinators (Woodcock et al., 2018). Additionally, neonicotinoid use in the rest of the world is expected to continue to grow (Simon-Delso et al., 2015) and residues have also been detected in 75% of honey samples from a global survey (Mitchell et al., 2017). For these reasons, the world's pollinators will continue to be exposed to sub-lethal doses of neonicotinoids for years to come. Therefore, we have a responsibility to continue to monitor their use and to understand how they can be used without harming pollinators and the wider environment, if at all.

The continued use of neonicotinoids and their wider environmental contamination is especially concerning because the majority of our understanding of the effects of neonicotinoid exposure on pollinators is based on studies of a single species: the western honeybee (*A. mellifera*) (Lundin et al., 2015). The relative paucity of information concerning the reactions of other pollinator species to neonicotinoid exposure has led to the development of pesticide risk assessment protocols that are unrepresentative of diverse pollinator communities (Stoner, 2016). Comparative work has shown that effects of neonicotinoid exposure can vary according to the specific test chemical and the pollinator species involved in the study (Cresswell et al., 2012; Moffat et al., 2016; Rundlöf et al., 2015). Honeybees may actually represent an especially poor model organism for predicting the level of risk faced by other pollinators. It is thought that the large colony sizes of honeybees (tens of thousands of workers) may afford additional resilience against environmental stressors, compared to less social species (Cresswell et al., 2012; Rundlöf et al., 2015). Additionally, the neonicotinoid detoxification rate of honeybees is relatively high (compared to bumblebees; Cresswell et al., 2012; Cresswell et al., 2013). Broadening the research effort to cover non-*Apis* pollinators is crucial if we are to understand the neonicotinoid exposure risk on wider pollinators communities.

The following section will review the evidence concerning the effects of sub-lethal neonicotinoid exposure on bumblebees (*Bombus* spp.), make comparisons with what is known about the effects on honeybees, and identify important knowledge gaps.

1.3 Bumblebees and Neonicotinoids

Bumblebees (*Bombus* spp.) are eusocial insects that (in temperate regions) live in annual colonies founded by a single mated queen that has overwintered from the previous year and can grow to contain up to approximately 400 workers (Benton, 2006). There are over 250 species distributed across the northern hemisphere, with some species found in South America (Benton, 2006). Across much of their range, bumblebees are vitally important pollinators of wildflowers and crops (Garibaldi et al., 2013; Klein et al., 2007; Ollerton et al., 2011). Colonies are also kept as managed pollinators and supplied to farmers to enhance the pollination of crops such as tomato and strawberry (Velthuis and Doorn, 2006). Pollination is carried out by a subset of non-reproductive workers from each colony that leave the nest to forage for nectar and pollen from flowering plants. Bumblebee colonies are exposed to neonicotinoids in the field when foragers collect nectar and pollen from treated crops and return it to the nest to provision the queen, the adult workers and the developing brood. This route of exposure constitutes a sub-lethal dose, but the multitude of effects of this sub-lethal dose on bumblebee physiology, behaviour, sociality, reproduction, populations and inter-specific interactions are still being described (Figure 1-1). In order to understand how and why these effects cause certain colonies to fail we must understand how the colony functions.

1.3.1 Bumblebee Biology

The buff-tailed bumblebee (*Bombus terrestris*) is one of the most common bumblebees in Europe and it is certainly the most intensively studied (Amsalem et al., 2015; Sladen, 1912); therefore, this species will be used as a

model to introduce bumblebee life histories. Additionally, all subsequent studies in this section describe *B. terrestris* unless otherwise stated.

Bumblebee queens emerge from hibernation in early spring and immediately begin to forage and to search for a suitable nest cavity in which to found a colony. Once a queen has found a suitable nest site, she will lay the first batch of eggs in a wax cup, where they will develop into larvae. During this stage the queen will continue to forage and to provision the larvae with pollen and nectar. The first of the queen's daughters begin foraging soon after emergence; they replace the queen as provisioners of the colony and after this point the queen will not leave the nest again. These workers will also perform a variety of other 'household' tasks within the nest such as incubating eggs, feeding larvae or cleaning the nest. The queen will continue to lay fertilised female eggs as the colony grows throughout the summer months. This is referred to as the *harmonious phase* and is characterised by rapid growth in the worker population and cooperation between workers and the queen in rearing more workers. The next significant event in the development of the colony is the *switch point*, which is when the queen switches from laying fertilised diploid eggs that will develop as females, to laying unfertilised haploid eggs that will develop as males. This switch triggers the enhanced feeding and development of the last remaining female larvae into reproductive daughter queens (gynes) (Duchateau and Velthuis, 1988). Once the gynes and males emerge as adults they leave the colony to mate. Mating results in the death of the males and causes the gynes to enter hibernation until the next spring. Towards the end of the colony lifecycle, the harmonious phase comes to an end as some workers with activated ovaries begin to interact aggressively with each other and the queen. The colony now enters a new phase, the *competition phase*, characterised by overt aggression

worker egg-laying (unfertilised haploid male eggs), oophagy (cannibalising worker-laid eggs), finally resulting in the death of the queen, who may be killed by the workers (Duchateau, 1989; Duchateau and Velthuis, 1989; Free, 1955a). Once the reproductive males and gynes leave to mate the colony condition declines and any remaining workers eventually die.

The ultimate mechanisms that produce such cooperative societies with a singly-mated queen are understood with reference to Hamilton's inclusive fitness theory, which states that these societies can evolve if workers gain more fitness benefits by helping to raise closely related kin than they would if they raised their own offspring (Hamilton, 1964). Under this framework we understand that bumblebee workers forgo individual reproduction and help to rear their sister gynes because the hymenopteran haplodiploid sex determination system results in higher relatedness between sisters ($r = 0.75$) than between workers and own daughter gynes, if they existed ($r = 0.50$). Thus, workers cooperate to help the colony grow and rear queen-laid gynes because of the relatively high indirect fitness benefits they gain in doing so. However, the competition point and worker egg-laying suggest that workers still attempt to gain direct fitness benefits given the right conditions. This is because workers are more related to their sons ($r = 0.50$) than they are to either their nephews ($r = 0.375$) or their brothers ($r = 0.25$) (Hamilton, 1972). Therefore, we expect workers to maximise their direct and indirect fitness benefits by competing with sisters and the queen over male parentage. The conflict tends to arise late in the colony cycle once worker investment in cooperation has resulted in the development of sister gynes (Alaux et al., 2005).

Queen-born gynes are the only individuals that form the next generation. In other words, the colony is the unit of reproduction and its reproductive

success depends on the combination of a productive queen, efficient worker cooperation across tasks, and sufficient growth in the worker population to provision energetically 'expensive' gynes (Amsalem et al., 2015). Given reports of recent declines in bumblebee populations in the industrialised world (Goulson et al., 2015), we must assume that contemporary environmental pressures are impacting one or more of these requirements of reproductive success. Neonicotinoid exposure experiments seem to suggest that these chemicals have the potential to disrupt each one of these requirements. However, many experiments also find no ill effects, and the causality between neonicotinoids use and bumblebee declines has not yet been proven. In order to affirm or deny this causal link we must continue to consolidate the results of such experiments and make more connections across levels of biological organisation from the cell to the community.

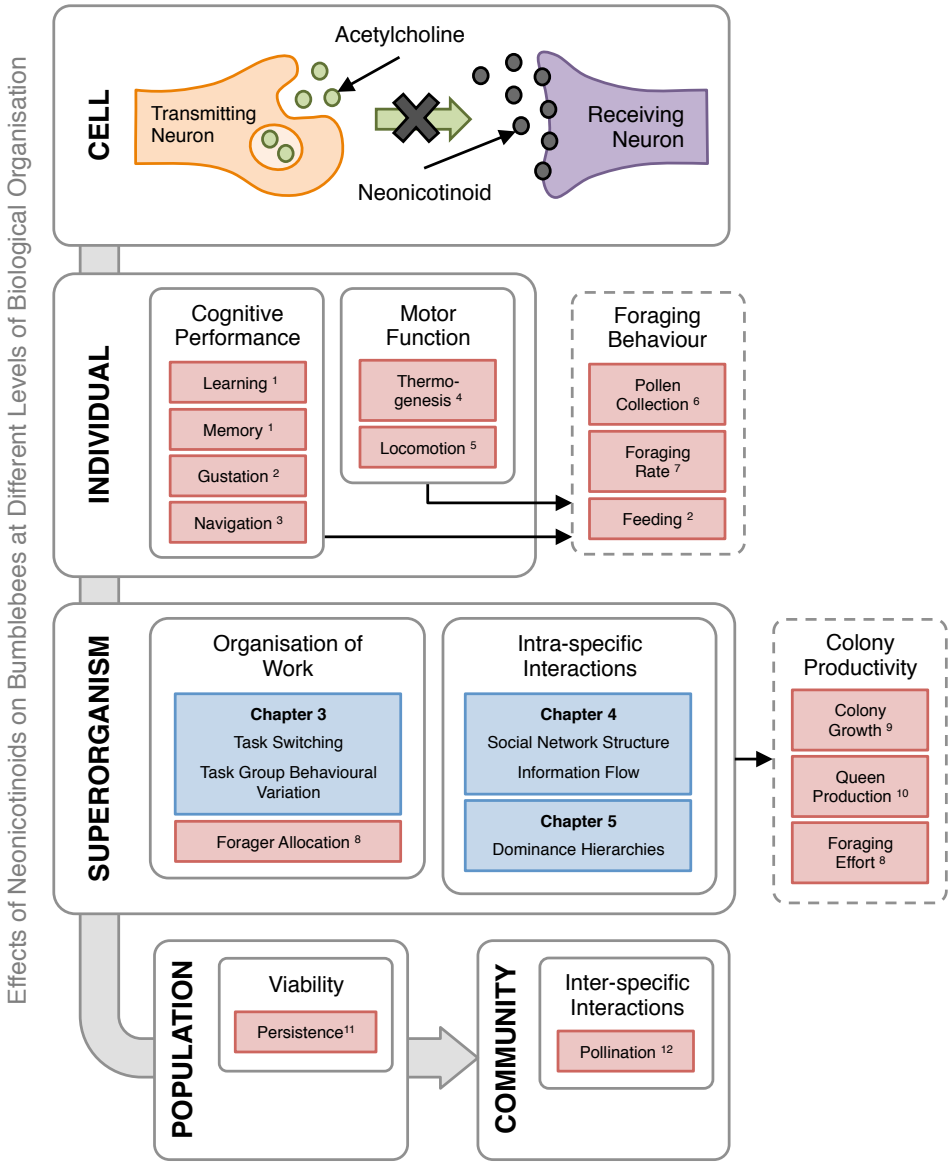

Figure 1-1. Effects of neonicotinoids on bumblebees at different levels of biological organisation. Red boxes show scientific evidence of negative effects of neonicotinoid (NEO) exposure on specific components of bumblebee biology. Red evidence boxes are grouped initially into functional categories, which are further grouped into the level of biological organisation at which they are observed (in order of increasing scale: Individual, Superorganism, Population, Community). Dashed boxes ('Foraging Behaviour' and 'Colony Productivity') represent categories of effects that are the net result of interactions between more fundamental categories of biological function (solid boxes). Blue boxes suggest

putative effects of NEO within fundamental mechanistic categories at the superorganism level that will be addressed in the labelled chapters of this thesis. 'Cell' panel depicts a simplification of the action of neonicotinoids as a acetylcholine receptor agonists, which blocks normal nervous transmission (Palmer et al., 2013). 'Individual' level shows evidence of effects of NEO on isolated individual insects or on the behaviour of specific individual insects living in groups/colonies. 'Superorganism' level shows evidence and sections of the current study that address the effects of NEO at the level above the individual, often referred to as the colony level. 'Population' shows effects of NEO on all the colonies of the same species that live across a certain geographic area. 'Community' shows effects of NEO on the relationship between the population of colonies and other species in a certain geographic area. References: 1. Stanley et al. (2015a); 2. Kessler et al. (2015); 3. Stanley et al. (2016); 4. Potts et al. (2018); 5. Cresswell et al. (2013) [high dose]; 6. Feltham et al. (2014); 7. Gill and Raine (2014); 8. Gill et al. (2012); 9. Rundlöf et al. (2015); 10. Whitehorn et al. (2012); 11. Woodcock et al. (2016); 12. Stanley et al. (2015b). Figure adapted from Alkassab and Kirchner (2016a) to specifically show only bumblebee-related evidence and to include the 'Superorganism' level.

1.3.2 Effects of Neonicotinoids at the Cellular Level

Neonicotinoids bind to acetylcholine receptors in the insect central nervous system and block normal acetylcholine nervous transmission. In bumblebees, three of the most widely used neonicotinoid chemicals [thiamethoxam (TMX), clothianidin (CLO) and imidacloprid (IMD)] are detectable at neuroactive levels in the brains of bees fed nectar containing 2.5 μgkg^{-1} pesticide over 3 days (Moffat et al., 2016). In the brain these chemicals target Kenyon cells, the primary neuronal cell type of the mushroom bodies of the bumblebee brain, where they each target specific receptor subtypes and ultimately cause neuronal deactivation (Moffat et al., 2015; Moffat et al., 2016; Palmer et al., 2013). The mushroom bodies are important brain structures known to integrate multisensory information and contribute to learning and memory in

bees and other insects (Heisenberg, 2003). Making this connection between the receptor-binding properties of specific neonicotinoid chemicals and the role of Kenyon cells in higher order brain structures of the central nervous system is the first step in piecing together how sub-lethal doses of these agricultural insecticides affect bees across multiple level of biological organisation (Figure 1-1). This physiological approach demonstrates the direct effect on the nervous system, and suggests the potential for effects on the muscular system, but to understand the extent of these putative effects on system functions we must study whole organisms.

1.3.3 Effects of Neonicotinoids at the Individual Level

1.3.3.1 Cognitive Performance

The proboscis extension reflex is a classic paradigm used to probe our understanding of insect cognition by interpreting the learning behavioural demonstrated individual organisms (Bitterman et al., 1983). In bees, this response involves the reflexive extension of the proboscis to feed when the antennae detect sweet nectar and can be paired with a stimulus for classical conditioning (Bitterman et al., 1983). Bumblebees are normally able to quickly learn to associate a stimulus with a nectar reward and will extend their proboscis in response to the conditioned stimulus alone; however, chronic exposure to TMX at 2.4 ppb significantly increased the number of trials taken to learn the association and negatively affected the memory retention of the association (Stanley et al., 2015a). These results strongly suggest that the action of neonicotinoids on the receptors of nerve cells can scale up to affect the nervous system and the behaviour of the individual. On the other hand, Piiroinen et al. (2016) found no effect of CLO at 1 ppb on

learning and memory in bumblebees, confirming that dose and chemical are significant factors.

Feeding behaviour more generally is also affected, albeit in more complex ways that are not yet fully understood. Bees (*B. terrestris* and *Apis mellifera*) are not able to taste low concentrations of neonicotinoids, but they prefer to feed on treated nectar over untreated nectar (Arce et al., 2018; Kessler et al., 2015) and often consume less nectar overall in exposure experiments (Kessler et al., 2015; Laycock and Cresswell, 2013; Laycock et al., 2012; Thompson et al., 2015). The nervous stimulation induced by the chemicals may affect feeding preference by interfering with learning food locations or with conditioning reward, whereas food consumption could be disrupted by effects on motor functions related to feeding (Arce et al., 2018; Kessler et al., 2015).

1.3.3.2 Motor Function

Monitoring the motor functions of individuals (and small groups of individuals) exposed to neonicotinoids has been an effective assay in understanding how basic functions of individual-level behaviour might be affected. Work on honeybees (*A. mellifera*) suggests a general dose-dependent effect on motor function ranging from hyperactivity at low acute doses, followed by increasing suppression of motor activity at higher chronic doses (Alkassab and Kirchner, 2018; Charreton et al., 2015; Lambin et al., 2001; Teeters et al., 2012; Tosi and Nieh, 2017; Williamson et al., 2014). In bumblebees, Cresswell et al. (2013) found that exposure to a nectar food source containing IMD at 98 μgkg^{-1} (much higher than typical field realistic concentrations) over 8 days reduced the distance moved by isolated individuals. By contrast, Cresswell et al. (2012) found no effect on the distance moved per hour of individually caged bumblebees after 4 days of

exposure to dietary IMD at a wide range of concentrations (0.0-125 μgkg^{-1}). Additionally, in the same study, Cresswell et al. (2012) found no effect of this exposure regime on honeybees in groups of 10 workers. Aside from locomotion, bumblebees (and other bees) also use their muscular system to produce heat by vibrating flight muscles (Esch et al., 1991). This crucial behaviour for pre-flight warming and brood incubation has been found to be affected by neonicotinoid exposure; IMD and TMX both induced dose-dependent reduction in warming rate (Potts et al., 2018). These somewhat mixed results generated from observing behaviours related to motor functions highlight the fact that predicting individual behavioural responses to neonicotinoids across assays, exposure regimes and species is an unresolved challenge.

1.3.3.3 Foraging Behaviour

Central-place foraging for nectar and pollen from spatially-distributed and temporally-available flowing plants is a significant cognitive and physical challenge for individual bees (Klein et al., 2017). As a result, successful individual foraging behaviour relies on integrating many fundamental components of individual physiology and behaviour. Neonicotinoid exposure appears to disrupt foraging behaviour by directly impacting some of these more fundamental components (Figure 1-1).

Given the importance of foraging for colony growth and reproduction, plus the high risk of pesticide-induced impairment to this complex task, foraging has been monitored as an end-point in many neonicotinoid exposure experiments. Once again, there has been a great deal of focus on honeybee foraging: with some negative effects (Henry et al., 2012; Ramirez-Romero et al., 2005; Tosi et al., 2017) and some neutral effects at doses/concentrations

considered to be field-realistic (Cutler and Scott-Dupree, 2007; Schneider et al., 2012; Thompson et al., 2016; Yang et al., 2008).

It seems *B. terrestris* foragers exposed to neonicotinoids are affected on a number of fronts including reduced number of foraging bouts (Gill and Raine, 2014), longer time spent foraging (Gill and Raine, 2014; Gill et al., 2012; Stanley et al., 2015b; Stanley et al., 2016), reduced pollen collection (Arce et al., 2017; Feltham et al., 2014; Gill and Raine, 2014; Gill et al., 2012; Stanley et al., 2015b), increased flower handling time [IMD 30 ppb] (Morandin and Winston, 2003) and reduced homing ability (Gill et al., 2012; Stanley et al., 2016). However, there are also reports of no effect on foraging rate for *B. terrestris* at IMD at 6 ppb in nectar and 0.7 ppb in pollen (Feltham et al., 2014), or for *Bombus impatiens* at 7 ppb in nectar (Morandin and Winston, 2003). In some cases, these reported negative effects on foraging behaviour affect the rate of food intake and appear to impact colony-level behaviour and performance (Section 1.3.4).

1.3.4 Effects of Neonicotinoids at the Level of the Superorganism

The term 'superorganism' is applied to describe how eusocial insect colonies (composed of individual insects) represent a distinct level of biological organisation above the individual, akin to how multicellular metazoans are distinct from single cells (Boomsma and Gawne, 2018). The way we often study the effects of neonicotinoid pesticides on social bee colonies is by recording colony-level outputs such as productivity, which provide important descriptions of *how* colonies are affected by exposure, but do not answer *why* (i.e. the mechanism by which) colonies are affected. Just as it is impossible to understand why neonicotinoids affect associative learning by measuring the proboscis extension reflex of individual bees, it is impossible to understand

why neonicotinoids affect superorganismal colonies by monitoring colony-level responses. The key to understanding why a bee will reflexively attempt to feed when presented with a conditioned stimulus is to dissect the individual's brain and track the patterns of interactions that occur between nerve cells during conditioning (Hammer, 1997). By analogy, the superorganism concept would suggest that in order to understand why bee colonies are affected by neonicotinoids we must dissect the colony and track connections between individuals. The aim of pesticide risk-assessment, with respect to social pollinators, is to be able to understand how colonies will respond to agrochemicals in the field and to make predictions based on our understanding (Domenica et al., 2016; EFSA, 2018a). Without a mechanistic understanding of how and why bee colonies are affected by pesticide exposure, our ability to make predictions is limited (Sponsler and Johnson, 2017). The remainder of this section will discuss the important contributions of colony-level studies to our understanding of how neonicotinoid exposure can affect colonies, highlight studies that begin to describe the responses of colonies from a superorganismal perspective, and suggest future directions for pesticide risk assessment research.

With increasing concerns about pesticide-induced declines in bee populations, the field of pesticide research has shifted from simple individual-level laboratory assays to incorporate 'higher-tier' risk assessment techniques. These techniques involve testing the responses of whole colonies in increasingly complex environments from the laboratory, to semi-field studies, to natural exposure in the field (Blacquière et al., 2012; Cabrera et al., 2016; Thompson, 2003). The end-points in higher-tier risk assessments tend to be measured at the level of the colony and may include colony growth, reproductive performance or pollination services (see Alkassab and Kirchner,

2016). The colony is the functional unit that reproduces, facilitates pollination, and is also somewhat resilient to the loss of individuals (Henry et al., 2015; Klein et al., 2017); therefore, this approach is well needed.

Steady growth in the bumblebee worker population is necessary for the colony to be able to reproduce successfully (Benton, 2006). Worryingly, decreases in bumblebee colony growth and reproductive output have been reported in several neonicotinoid exposure experiments. Whitehorn et al. (2012) exposed queenright colonies to IMD at 6 ppb and 0.7 ppb in nectar and pollen, respectively, for 2 weeks in the laboratory before allowing them to develop naturally in the field. Treated colonies suffered significantly reduced growth rates (colony weight) and an 85% reduction in queen production. Since then, two large replicated field studies have found similar results. In Sweden, Rundlöf et al. (2015) described reduced growth rate and queen production in bumblebee colonies adjacent to fields of oilseed rape grown from CLO-treated seeds. Woodcock et al. (2017) monitored bumblebee colonies adjacent to oilseed rape fields treated with either CLO or TMX across German, Hungary and the United Kingdom. They found no direct effect of field treatment on bumblebees, but found a negative correlation between egg cell production and the concentration of neonicotinoid residues sampled from nest material. The residues contained CLO, TMX and IMD, suggesting wider pollution of these chemicals had contaminated the colony (Woodcock et al., 2017), possibly via wildflowers (Botías et al., 2015; David et al., 2016).

These field studies provide strong evidence that bumblebee colonies are negatively affected by field-level exposure to neonicotinoids. However, they do not attempt to test the mechanisms behind observed effects. The best example of a study attempting to test these mechanisms was a semi-field

study by Gill et al. (2012). These authors provided early stage colonies (queen plus 10 workers) with access to nectar containing IMD at 10 ppb via a feeder inside the nest for 4 weeks. Colonies were housed in the laboratory but were required to forage outside for pollen. This study found that IMD exposure reduced colony growth (workers production and number of brood), and increased worker loss (mortality and losses outside). The novelty of this study was the parallel measurements of individual foraging bouts during exposure. They found that individuals in treatment colonies performed longer foraging bouts and collected less pollen than individuals in control colonies, suggesting nutrient limitation as an explanation for reduced colony growth. The really interesting result however, was an increase in the number of foragers in treatment colonies, interpreted as "response to reduced individual foraging efficiency". Yet, Gill et al. (2012) list this increase in the number of foragers within the category of "effects on individual behaviour". Experimental work (not related to pesticides) has shown that the allocation of more workers to foraging is actually a collective colony-level response to reduced individual foraging efficiency that emerges as a result of information exchanged via interactions between foragers, non-foragers and nectar pots; eventually, this information influences the decision of certain non-foraging workers to leave the nest in search of food (Crall et al., 2018; Dornhaus and Chittka, 2001; Dornhaus and Chittka, 2005; Renner and Nieh, 2008). Therefore, this effect should be categorised as an effect at the level of the superorganism (Figure 1-1).

The categorisation of effects at the "level of the superorganism" is not just semantics, nor does it challenge the findings of Gill et al. (2012). This perspective attempts to conceptualise colony behaviour in a way that can enhance our mechanistic understanding of the system and its responses. The

superorganismal response of reallocating workers to foraging (presumably at the expense of other tasks) in order to compensate for low food intake is a complex behaviour that requires a high level of integration between individuals and demonstrates the resilience and flexibility of the colony. Indeed, comparative studies seem to suggest that higher levels of sociality confer additional levels of resilience against the effects of pesticide exposure and other environmental stressors (Klein et al., 2017; Rundlöf et al., 2015; Woodcock et al., 2017). It seems we often assess pesticide exposure risk by recording the responses of colonies as if they were outputs from a black box whose internal workings are concealed from us. This approach does not make use of the fact we can track and manipulate the individuals that make up entire superorganism systems (Kennedy et al., 2017). If we are to understand how and why neonicotinoids affect colonies and the test the limits of social resilience we need to incorporate an understanding of the colony as complex biological system exhibiting organisation and behaviour distinct from a simple summation of the behaviour of individuals.

1.4 A Complexity Science Approach

The Newtonian approach to scientific thought attempts to describe the world through reductionism. By creating idealised models of the world, this approach attempts to provide general law-like mechanisms to explain the individual parts of observable phenomena. Complexity science, on the other hand, is a different approach that views the world as an open system composed of many interconnected elements, whose relationships give rise to complex collective properties (Bar-Yam, 1997a). This approach of embracing complexity, instead of minimising it, has advanced our understanding of the properties and behaviour of many complex real-world systems such as

economies, multi-cellular organisms and the Internet. These examples are not just complicated patterns; they are specifically referred to as *complex systems* (Holland, 2014).

The key property exhibited by a complex system is called *emergence*, which can be summarised by the phrase 'the whole is greater than the sum of its parts'. This common phase describes the situation where a number of individual components of a system produce complex behaviours as a collective that cannot be attained by a summation of individual behaviour. The encoding and replication of genetic information via DNA molecules is an example of an emergent property of the molecules inside a cell (Morowitz, 1995). None of the individual cellular components show any capacity to self-replicate. However, the interactions between enzymes, proteins and specific sequences of nucleotides produce a complex system that facilitates reproduction in all known living organisms. Emergence in complex systems gives rise to new hierarchical levels of organisation that can further interact as components of systems at different scales (Bar-Yam, 1997b). DNA molecules interact with enzymes to replicate cells, cells interact to form multicellular organisms, organisms interact within populations, etc. This scaling of complexity in hierarchical systems is said to be *non-linear* because high orders of organisation do not emerge from the summation of a systems components (Bar-Yam, 1997b).

The mechanism behind the emergence of organisation in complex systems is often understood as a process of *self-organisation*. This suggests that the global-level coordination that we observe in complex systems is not dictated by some external entity, but occurs as a result of processes internal to the system. The process is spontaneous and is triggered by random fluctuations in the interactions between components that become amplified by feedback

loops, ultimately generating collective patterns from local interactions (Camazine et al., 2001). The emergence of the dynamic shapes formed by a flock of starlings are not directed by a leader, but are the result of each bird reacting to the flight of its neighbours (Hildenbrandt et al., 2010). The study of the mechanics of self-organisation is central to the way we study and understand the behaviour of complex systems in general (Camazine et al., 2001).

One of the most important analytic tools used to study complexity comes from modelling systems as *networks*. This tool is derived from *graph theory*, which is a branch of mathematics that is interested in the properties of different configurations of pairwise relations between objects (Barabási, 2016). This set of objects and relations can be modelled as a network of *nodes* (the objects) connected by *edges* (their relations). The graph theory approach (also known as *network theory*) of connecting pairwise interactions between components into a larger scale network is well suited to be able to characterise the local and global properties of systems of interconnected units, such as the social relations between groups of animals (Croft et al., 2008). In the late 1990s, the discovery that the interactions between components of real-world systems often exhibit common network-level properties has since shaped our understanding of the fundamental features of complex systems (see Barabási, 2016). One common property of most real-world networks, such as The Internet, scientific co-authorship networks and protein-protein interaction networks is the occurrence of a small number of nodes with many connections, while the majority of the nodes have very few connections (Barabási, 2016). For this kind of network, the distribution of the number of connections per node (*degree*) follows a power law and the network is said to be *scale-free* (Barabási et al., 1999). Simulating the removal of random nodes

from scale-free networks has shown high robustness of the structure (*topology*) and function of these networks (Albert et al., 2001). The prevalence of scale-free networks across such different systems shows the power of network theory to describe complexity at vastly different scales.

Returning to the superorganism concept, we can see that social insect colonies share many features with complex systems: they are composed of interacting individuals, they exhibit the emergence of complex behaviours via processes of dynamic self-organisation, and they represent a level of organisation that is distinct from the individual (Bonabeau, 1998). Indeed, over the past 40 years, the complexity science approach led to significant advances in our understanding of the complex behaviour of superorganisms, including: flexible task allocation (Gordon, 1996; Gordon, 2002a; Robinson et al., 2009b), nest construction and homeostasis (Bonabeau et al., 1998; Collins, 1979; Weidenmüller et al., 2002), foraging trial formation (Couzin and Franks, 2003; Franks and Fletcher, 1983; Franks et al., 1991) and collective decision-making (Feinerman and Korman, 2017; Franks, 1989). However current neonicotinoid exposure research has not yet made use of the advantages of a complex systems approach to understand colony failure. This thesis will approach the problem of understanding the effects of neonicotinoids on bee colonies from a complexity perspective to provide novel insights that would not be possible from considering either effects on individuals or the average of colony effects.

1.5 Aims

The aim of this thesis was to examine the effects of chronic sub-lethal neonicotinoid exposure on bee colonies by considering the individuals within the colony as integrated components of a complex system. To achieve this

aim automated, high-throughput video tracking techniques were implemented to track the behaviour of entire queenright bumblebee (*Bombus terrestris*) colonies in the laboratory (Chapter 2). High-resolution tracking data was used to automatically describe individual behaviour in a social context before, during and after neonicotinoid exposure (Chapter 3 and Chapter 4). Additionally, detailed manual recording of directed dominance interactions was used to track the effects of neonicotinoid exposure on the formation of dominance hierarchies (Chapter 5).

1.5.1 Chapter 2: Employing automated techniques to enhance the collection of bumblebee behavioural data during pesticide exposure experiments.

To date, assessments of the risks associated with exposure of social bees to neonicotinoid pesticides has focussed on testing the *average* response of isolated individual bees, small queenless groups or full queenright colonies; this approach attempts to reduce social complexity by measuring simple endpoints (see Alkassab and Kirchner, 2016a). Measuring average colony-level responses can identify symptoms of neonicotinoid exposure, but cannot reveal the underlying mechanisms from which social complexity emerges. The aim of Chapter 2 was to make use of recent advances in video tracking software to capture the complexity of whole bumblebee colonies by automatically tracking the behaviour of every single individual. Complete colony tracking will greatly improve on the current techniques in pesticide research by integrating individual-level responses within colony-level system responses. An open-source video tracking software package (BEEtag, Crall et al., 2015) was customised specifically for use in this chapter to enhance the performance of tracking ultra-high resolution video inside bumblebee colonies. Furthermore,

Chapter 2 describes the complete step-by-step procedure developed specifically to convert raw tracking data into accurate high-quality bumblebee behaviour data. This procedure includes methods to identify and correct sources of error, produce detailed measurements of locomotor behaviour, and automatically record social interactions between tracked bees. The result was an incredibly rich dataset that was used to test the effects of pesticide exposure on inter-individual behavioural variation and task allocation (Chapter 3), and on the structure and function of dynamic social interaction networks (Chapter 4).

1.5.2 Chapter 3: Are all individuals within a colony equally affected by neonicotinoid exposure?

The effects of neonicotinoid exposure on bees have been described by many different assays and end-points (see Alkassab and Kirchner, 2016a), but the effects of exposure on the behaviour of all individuals within the social context of a functioning colony has not been tested (Heimbach et al., 2017). Within colonies of bumblebees, individual workers exhibit great variation and plasticity in their behaviour, including movement speed, space use, and foraging activity (Crall et al., 2018; Jandt and Dornhaus, 2011; Jandt et al., 2009; Martin et al., 2018; van Honk and Hogeweg, 1981; Weidenmüller, 2004; Woodgate et al., 2017). Given that groups of workers behave differently and experience different social and environmental conditions, should we expect all individuals to respond to neonicotinoid toxicity in the same way? Chapter 3 utilised the high-resolution video tracking methods and data from Chapter 2 to track changes in key metrics of locomotor behaviour related to task allocation (plus foraging activity) before, during and after neonicotinoid exposure. The results show that social context does mediate the effects of

neonicotinoids on basic locomotor behaviours, with differential effect correlating to the extent of individual foraging activity (including foraging inactivity). As well as demonstrating the risks of socially-mediated individual-level pesticide exposure, this chapter suggests that future pesticide risk assessment studies must take social context into account and measure the many complex and inter-related components of behaviour simultaneously, or else effects on certain social groups could go unnoticed.

1.5.3 Chapter 4: How do individual-level effects of neonicotinoid exposure scale up to affect the social network?

Social insect colonies share many characteristics of complex systems: they are made up of many interdependent interacting components and they exhibit the emergence of global-level behaviour and properties via self-organisation (Bonabeau, 1998; Charbonneau et al., 2013; Gordon, 2002a). The emergence of properties such as the organisation of work or colony-wide information flow are said to be complex because their collective outcome is not achieved by a simple summation of the actions of many individuals. These properties are characterised by nonlinear emergence, i.e. they occur as a result of the interactions between individual components (which can scale exponentially with the number of components). Network analysis can be used to quantitatively describe the structure and dynamics of the intricate networks of pair-wise interactions within real-world complex systems such as social insect colonies (Charbonneau et al., 2013; Fewell, 2003; Naug, 2015). Chapter 4 aims to use this analytic approach to describe how individual behavioural impairment during neonicotinoid exposure (Chapter 3) scales up to affect emergent colony-level processes. It has been difficult to reconcile individual-level effects and colony-level effects of neonicotinoid pesticide exposure

research because we do not understand the mechanics of non-linear scaling in exposed bumblebee colonies (Henry et al., 2015; Sponsler and Johnson, 2017). The network approach of Chapter 4 addresses this issue directly.

Chapter 4 aims to utilise the trajectories of individual bees generated via video tracking to automatically detect social encounters. Chapter 2 describes the technical details behind two types of contact-based social interactions: a simple proximity interaction, and a more spatially explicit measure of head-to-head proximity interactions (which approximates to antennation). By constructing networks of these interactions in control and treatment colonies it is possible to track changes in the structure and dynamics of bumblebee colony social networks over time and during pesticide exposure. Additionally, these networks are used to model task-group mixing patterns and information flow, which represent emergent colony-level network processes. The results find evidence of changes in network structure that can be explained with reference to the behavioural effects described in Chapter 3. However, it appears as though the effects on networks structure do not scale up to detectable effects on network processes, suggesting unexpected social resilience at the level of the colony.

1.5.4 Chapter 5: Can neonicotinoid exposure disrupt social dominance structure?

One of the big outstanding questions in our understanding of the impacts of neonicotinoids on bees is exactly how exposure causes a decrease in brood production (Gill et al., 2012; Laycock et al., 2012; Laycock et al., 2014). It has been suggested that a disruption in the social interactions that stimulate egg-laying in queenless groups of bumblebees (microcolonies) could explain reductions in the number of brood (Laycock et al., 2012). The aim of chapter

5 was to test for an effect of neonicotinoids on the social interactions within microcolonies. If such interactions are affected, the findings could help to explain how brood production is affected in queenright colonies as well.

The primary aim of Chapter 5 was to incorporate the idea of socially mediated pesticide exposure risk (Chapter 3) with the idea of the emergence of social structure from interactions (Chapter 4) and test the effects of neonicotinoid exposure on a measurable property of social structure: the bumblebee dominance hierarchy. In certain conditions, bumblebee workers engage in agonistic interactions that affect individual ovary development and ultimately lead to the self-organising formation of a linear dominance hierarchy (Amsalem et al., 2013; Bloch and Hefetz, 1999; Duchateau, 1989; Free, 1955a; van Honk et al., 1981). This process relies on inter-individual variation and social interactions, both of which can be disrupted by neonicotinoid exposure (Chapter 3 and Chapter 4). One of the specific aims of Chapter 5 was to test if an individual's position in the dominance hierarchy affected the toxicity of the pesticide. Further to this, would neonicotinoid exposure disrupt the relationship between reproductive dominance (based on ovary development) and behavioural dominance (based on agonisitic interactions)? The results show that exposed bees engage in fewer agonistic dominance interactions and the effect is the strongest on the most dominant individual. However, the relationship between reduced interactions and ovarian development is not clear and further work is needed to understand exactly how brood production is limited by neonicotinoid exposure.

Chapter 2

General Methods

2.1 Introduction

Manual collection of behavioural data by a trained observer has been the standard approach in behavioural ecology for decades, but the product of this approach is limited in terms of accuracy, precision and scale. Modern approaches to behavioural data collection are evolving rapidly as technological advances provide biologists with new tools to answer questions that were previously beyond study (Dell et al., 2014). Image-based tracking in particular is becoming increasingly popular, as shown by the regular publications of new tools (e.g. Gernat et al., 2018; Rodriguez et al., 2018; Yamanaka and Takeuchi, 2018), and has led to significant advances in the study of animal kinematics, collective behaviour and social organisation (see references in Dell et al., 2014). The benefits of image-based tracking include the ability to use the generated trajectory data to measure many components of individual behaviour simultaneously within the same system and at high spatiotemporal resolution. Trajectories describe the change in the positions of animals through space and time; therefore they can be used to describe many components of behaviour such as locomotion, space-use, sociality, and circadian rhythms (e.g. Crall et al., 2018). Approaches to image-based

tracking can be divided into two broad categories: feature-based detection and tag-based detection. Feature-based detection approaches aim to differentiate individuals directly from their representation in an image by using a combination of image processing techniques and trajectory analysis (Branson et al., 2009; Pérez-Escudero et al., 2014; Rodriguez et al., 2018; Yamanaka and Takeuchi, 2018). This approach can be powerful for tracking individuals or groups, but is dependent on even lighting against a visually homogeneous background. The alternative is a tag-based approach, which relies on very similar image processing techniques, but they are used to detect unique visual tags attached to the animals (Crall et al., 2015; Gernat et al., 2018; Greenwald et al., 2015; Mersch et al., 2013; Nagy et al., 2013). Marking animals with tags is invasive and could disrupt behaviour, but this approach can be used to track animals in any complex environment.

At the advent of the modern approach to sociobiology, Wilson (1971) wrote (of social insects) that "the reconstruction of mass behaviour from a knowledge of the behaviour of single colony members is the central problem of insect sociology." Automated tracking techniques have been widely adopted in social insect research because the possibility to track all the components of this complex biological system is a significant step toward addressing this challenge. Image-based tracking is particularly well suited to tracking social insect colonies because both individuals and colonies can be manipulated to ensure tracking is able to function effectively. This new automated approach in social insect research has demonstrated its value understanding the organisation of task allocation (Crall et al., 2018; Mersch et al., 2013), long-term activity cycles (Beer et al., 2016; Meshi and Bloch, 2007), and the structure and function of interaction networks (Gernat et al., 2018;

Otterstatter and Thomson, 2007; Pinter-Wollman et al., 2011; Richardson et al., 2017).

The advantages of these automated approaches have also been applied to monitoring and understanding the subtle behavioural effects of sublethal pesticide exposure on social bees. Video-tracking honeybees (*Apis mellifera*), in small groups, has revealed locomotor deficits induced by exposure to neonicotinoids (Alkassab and Kirchner, 2018; Charreton et al., 2015; Teeters et al., 2012). There is strong evidence that neonicotinoid pesticides can affect the behaviour of isolated individual bees and also the functioning of whole colonies, but our understanding of the exact mechanisms that cause colonies to fail is incomplete (see Alkassab and Kirchner, 2016a). In order to fully understand the level of risk posed by neonicotinoid exposure to bee colony functioning, we must track individual-level behaviour *and* colony-level behaviour within functioning colonies. This thesis addresses this need by taking a 'reality mining' approach by utilising machine-sensed data to describe complex social behaviour (Krause et al., 2013), in order to fully understand the effects of neonicotinoid exposure on bee colonies.

The relatively small size of bumblebee colonies (maximum ~400 workers) and their importance as pollinators in agricultural landscapes (see Chapter 1) makes them an ideal model system to make use of the potential power of automated tracking to detect effects of pesticide exposure across both individuals and colonies . Recently, the tag-based approach to visual tracking has been shown to be effective in tracking queenright bumblebee colonies (Crall et al., 2015; Crall et al., 2018). The BEEtag system used in these studies will be employed here because it is tag-based, free, open-source, and tested on bumblebee colonies.

The aims of this chapter were to implement high-resolution video tracking of entire bumblebee colonies (*Bombus terrestris*) and to automate the collection of high-quality behaviour data that could be used in a controlled and replicated colony-level pesticide exposure experiment (presented in Chapter 3 and Chapter 4).

Glossary

centroid
: The encoded centre of the tag.

blob
: A **B**inary **L**arge **OB**ject, in image processing terms, is a group of pixels with the same pixel values.

frame
: A single static image taken from an image sequence or video.

hamming distance
: The number of positions that are represented by different symbols when comparing two strings (e.g. character strings, binary strings, etc.).

parity bit
: An extra bit in a string of binary code that stores information related to the number of 1-bits in the string. There are two types of parity bits: an even parity bit is one if the number of 1-bits is even; an odd parity bit is one if the number of 1-bits is odd. The parity bit is used to error-check the string.

tag
: The physical label attached to the animal that carries a unique identification code.

tag detection
: The digital representation of a tag that has been automatically identified in an image. May be described as 'true' if the tag detection accurately represents the information encoded in a tag within the image, or 'false' if does not.

thresholding
: The process of converting a greyscale image to a binary image.

trajectory
: A sequence of data points describing movement through space and time.

2.2 Guide to This Chapter

The methods in this chapter were developed to generate a high-quality video tracking data set from video and images of bumblebee behaviour recorded according to the experimental design of Chapter 3 and Chapter 4. The data collection was the same for both of these chapters and will be described here in brief before the full technical details are provided in the next section.

A total of 10 colonies (Colony F, G, H, I, J, K, L, M, N and O) were kept in laboratory conditions and monitored for 19 days. Each colony began the experiment with a queen, 50 workers, and brood. All adults were marked with tags from the BEEtag system (see Section 2.2.2). Colonies lived in small artificial nest boxes (see Figure 2-2). Foraging bees were able to leave the nest via a tube connected to a larger foraging arena, where they could fly and collect nectar from a feeder (see Figure 2-3). Behavioural sampling was conducted for 1 hour each day. Sampling involved: 1) video recording the inside of the nest from above, and 2) motion-triggered image recording of forager activity at the nectar feeder (see Section 2.2.4). This image-recording procedure was used to generate 178 hours of intranidal video and 860,242 feeder images. This video/image dataset forms the input data for this Chapter. The subsequent sections describe how this video/image data was processed using the BEEtag system to generate tracking data (see Section 2.4), how the tracking data was cleaned (see Section 2.4), and finally how the tracking data was used to interpret bumblebee behaviour (see Section 2.5).

Figure 2-1. Still image taken from one of the intranidal videos. Bees are individually marked with unique visual tags from a customised version of the BEEtag video tracking

2.2.1 Bumblebee Set-up and Data Collection

2.2.2 Marking Bees

Tags generated by the BEEtag software were printed at high resolution (1200 dpi) on durable waterproof paper (Toughprint® A4). Tags were coated with a protective layer of clear nail polish (Collection® 2-in-1 Top and Base Coat) before being cut out individually with scissors. Bees were individually held in a marking cage (a plastic cylinder with a plunger inside to hold the bee against the mesh covering on one end) while a tag was stuck onto the middle of the thorax with a drop of super glue [Colony F and G: Loctite® Super Glue Liquid (not recommended); Colony H, I, J, K, L, M, N and O: Loctite® Super Glue Gel (recommended)]. The size of each printed tag was 3 mm per side, which was small enough to fit on the thorax without disrupting the wings. The encoded 'front' of the tag was aligned to the anterior end of the bee. Marking in this way did not seem to affect the behaviour of the bees, except an increase in self-grooming immediately after marking. Crall et al. (2015) found no effect of BEEtag marking on bumblebee mortality.

Regularly cleaning tags was also a necessary step in ensuring reliable tag detection. The tags of all marked bees were inspected daily. If any tags had any material (e.g. nest wax) obstructing the visual code, the bee was briefly removed from the colony and the tag was wiped clean (with a finger).

2.2.3 Nest Box Design

All experiments were conducted in a laboratory setting, which required colonies to be housed in artificial nest boxes. Nest boxes were designed to facilitate recording high quality video of the colony that would be amenable to BEEtag tracking. The nest boxes were constructed from transparent

acrylic and the dimensions were 180×100×100 mm (Figure 2-2). This size was smaller than nest boxes supplied by BioBest N.V., Belgium (280×200×180 mm) but was large enough to fit a single colony for the duration of the experiment and was within the range used by other studies (Pomeroy and Plowright, 1980; Sladen, 1912). The relative dimensions of the base of the nest box were specifically designed to adhere to the 16:9 aspect ratio of commercial video cameras. These dimensions maximise the video coverage of the nest when filmed from above.

The nest box was constructed from transparent acrylic to allow the nest contents to be lit from the sides by 4 purpose-built panels of red LEDs (Kingbright L-7104SRC-D, 640nm; see Figure 2-2). Red LEDs were used because bumblebees are not sensitive to red light (Peitsch et al., 1992); therefore, the panels should cause little disruption to bumblebee behaviour during video recording. Furthermore, the nest box and the lights were housed within a larger cardboard blackout box (Figure 2-2) to maintain darkness inside the nest.

2.2.4 Image Recording

Video-recordings of the inside of the nest were made with an ultra-high resolution (2160×3840 pixels) SONY FDR AX-100 camcorder at 25 frames per second. The camera was positioned directly above the nest box, looking down through the glass lid into the colony (Figure 2-3). A camera was fitted with a shade to block external light from entering the nest.

Foraging behaviour was monitored by recording still images of bees with a Logitech C920 USB webcam positioned above the feeder in the foraging arena (Figure 2-3). A Raspberry Pi 3 (Raspberry Pi is a trademark of the Raspberry Pi Foundation) computer controlled the webcam and recorded

high-definition images (1080×1920 pixels) directly to an external hard drive whenever motion was detected in the frame, at a maximum rate of two frames per second. Motion detection was controlled by the open source software motion© (https://motion-project.github.io/). At the beginning of each observation period the time and date settings of the nest camcorder and the Raspberry Pi controlling the feeder webcam were synchronised. Due to the sporadic recording of feeder images by motion detection, the images were each saved with a unique timestamp for later reference.

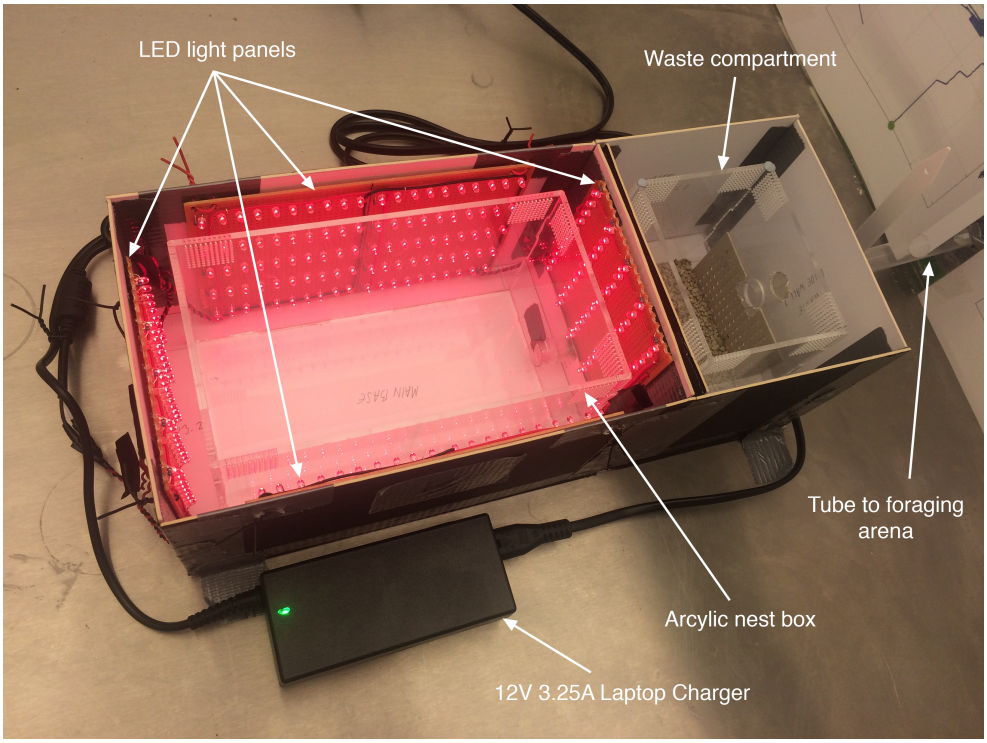

Figure 2-2. Bumblebee nest box for intranidal video recording. Nest box is shown open without the camera or camera shade in place. LED light panels are powered by a 12V 3.25A laptop charger. The waste compartment vestibule is attached to the foraging arena by a short length of tube.

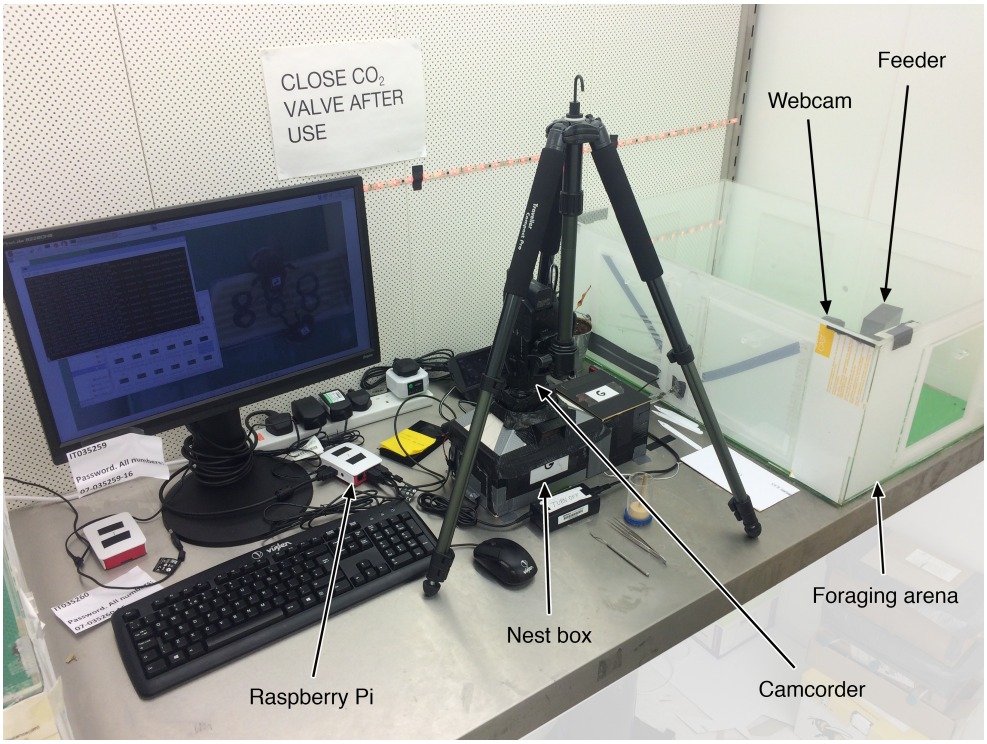

Figure 2-3. Set up for video tracking bumblebee colonies in the lab. Photograph of the equipment in place for recording intranidal bumblebee behaviour plus external forager behaviour. Colonies were housed in the nest box. The camcorder recorded video footage of the inside of the nest. A Raspberry Pi controlled the webcam, which recorded pictures of the feeder inside the foraging area onto an external hard drive (webcam feed displayed on the computer monitor).

2.3 Generating Video Tracking Data

2.3.1 Visual Tracking System

BEEtag is a free, open-source visual tracking system based on detecting barcode-like tags in images or video (Crall et al., 2015). The system is comprised of a small library of functions that operate within the MATLAB programming environment. There are two main functions; one function is used to generate digital files of the tags (that can be printed onto paper), while the other is used to locate the tags in an image ('`locateCodes`') and save the identity, location and orientation of each. The tag design is based on a 5×5 binary matrix that can store 25 bits of information (Figure 2-4). For the current study, all of the BEEtag functions were customised to generate and locate simplified tags with a 4×4 binary matrix to encode 16 bits of information (Figure 2-5). The customisation involved editing the default MATLAB scripts and deciding on a new error check design (see Figure 2-4 and Figure 2-5). This simplified 16-bit version was preferred because early tests of the 25-bit version within the specific experimental set-up of this study produced disappointing results in terms of the rate of tag detection. Simplification can increase the rate of detection, but at a cost of reduced tag diversity and increased false positive rate (see below). The overall functionality of the 25-bit and 16-bit tag design was the same, but the specifics of the 16-bit tags will be discussed here, while the full details of the 25-bit can be found in Crall et al. (2015). All of the bundled 25-bit BEEtag functions were customised to generate and detect the new 16-bit tags.

When the 16-bit tags were generated by the customised BEEtag functions, the information stored within each tag was encoded as a 4×4 grid of black and white pixels, surrounded by a white pixel border and a black

pixel border (Figure 2-5). This matrix of pixels encoded 16-bits of information, divided into a 12-bit identification code and a 4-bit error check. The identification code was encoded column-wise within a 4×3 section of the tag pixel matrix as a 12-digit binary number left-padded with zeros (Figure 2-5). The error check was encoded within the final 4-digit column of the matrix. The first three digits of the error check encoded the even parity (1 (white) for an odd number of 1s; 0 (black) for an even number of 1s) of each of the three columns of the identification code. The final digit encoded the parity of the entire identification code. The number of unique identification codes that can be generated from 12 bits is 4096. The number of codes was limited to only those that could operate in a single orientation, which left 3368 unique codes. The final list of codes each had a hamming distance of 2 relative to all other codes, which left 328 robust codes used in the experiment.

The function to locate 16-bit tags in an image operated according to a few simple image-processing steps, plus the additional steps needed to read the 4×4 binary grid. The first step in image processing was to threshold the image, thus converting it to a binary image. The `locateCodes` function then used a built-in MATLAB function '`regionprops`' to detect contiguous white blobs (see Glossary) and record basic morphological features of each one. The black outer border of each tag isolated the inner white region from the background, which allows tags to be easily detected by this step. Any blob that was within a user-defined size range (pixel area) and had four corners (according to the BEEtag function '`fitquad`') was stored as a potential tag. Within each 4-cornered blob, the `locateCodes` algorithm attempted to read the 4×4 binary matrix by comparing the identification code and error check in each orientation. If the error check confirmed the parity of the identification code and the code was part of the library of used codes, the

identification number, position and orientation of the tag were all saved in a MATLAB structure array.

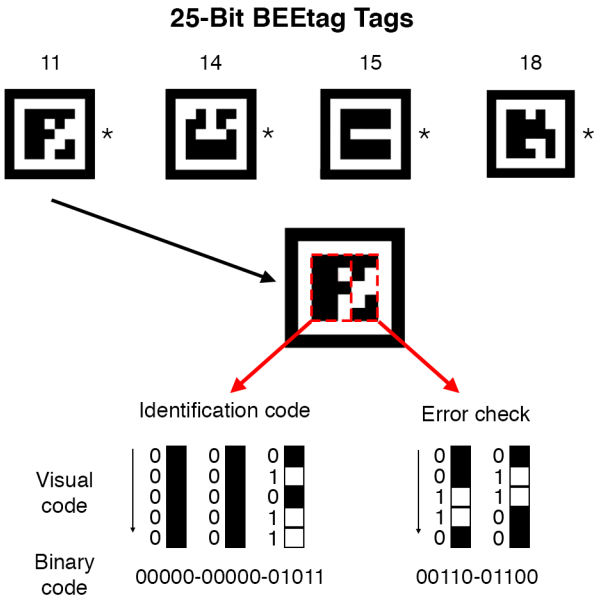

Figure 2-4. 25-bit BEEtag design. Four examples of 25-bit BEEtag tags displayed with the identification number above and the asterisk denoting the encoded 'front' orientation of the tag to the right. Tag #11 enhanced with red dashed boxes to highlight the identification code on the left and the error check on the right, which are dissected below to show how the binary number strings are encoded within the binary matrix. See Crall et al. (2015) for full details of the 25-bit tag error check technique. Figure adapted from Crall et al. (2015).

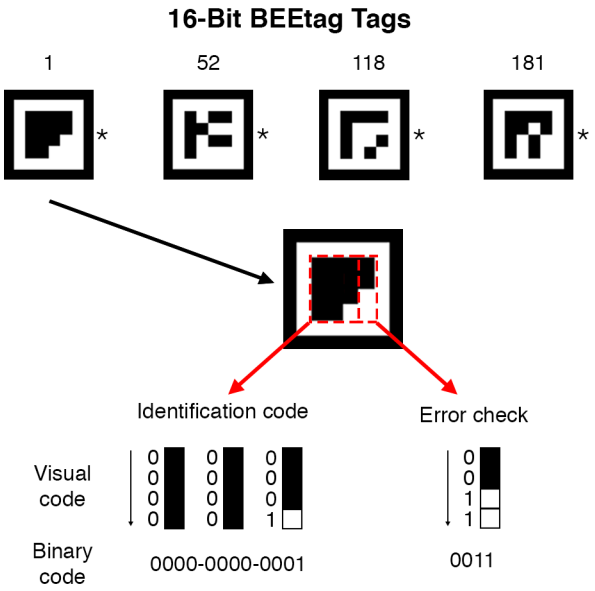

Figure 2-5. 16-bit BEEtag design. Four examples of 16-bit BEEtag tags displayed with the identification number above and the asterisk denoting the encoded 'front' orientation of the tag to the right. Tag #1 enhanced with red dashed boxes to highlight the identification code on the left and the error check on the right, which are dissected below to show how the binary number strings are encoded within the binary matrix. See Section 2.3.1 for description of the 16-bit tag error check technique. Figure adapted from Crall et al. (2015).

2.3.2 Factors Affecting Tracking Performance

In the paper that demonstrated the use of the BEEtag system, Crall et al. (2015) showed that the size of the tag in the image (the mean pixels per tag side), the image noise, and the threshold intensity level of the `locateCodes` function settings each had a significant effect on tag detection performance. The effect of each of these factors will be specific to the exact image-recording set-up used, but the general principals of each and the approaches used to address them in this study will be descried below.

The number of pixels per tag side was determined by a combination of the physical size of the tag, the resolution of the camera used, the tag distance from the camera and the focal length of the camera lens. For 25-bit tags, 25 pixels per side was suggested as a minimum size, below which tracking performance dropped significantly (Crall et al., 2015). This value was used as a benchmark during the design of the current image-recording set-up. The result of the tag size, video camera and nest box size used in the current study produced images inside the nest with approximately 40 pixels per tag side, which provided a good starting point for reliable tag detection.

Image noise (grain) can be affected by many components of the image recording process, but perhaps the most important setting for digital camera systems is the ISO (the camera's light sensitivity). High ISO is used to increase the brightness of an image, but will also increase noise. Therefore, light levels are important for regulating image noise. The lighting used in the current study was designed to provide maximum luminance for image recording, while reducing the heat generated by electric lights and limiting the potential disturbance to bumblebee behaviour (colonies are found naturally in dark subterranean nests; Benton, 2006).

The option for local adaptive bradley thresholding (a thresholding technique, see Glossary) in the BEEtag `locateCodes` function was used because it can account for uneven lighting and produce better images for analysis in certain conditions (Crall et al., 2015). The exact settings for bradley thresholding (filter size & threshold value) were optimised by tracking tags from a sample of images, each processed at a range of filter sizes & threshold values. The combination of setting that detected the most tags was: filter size = [15, 15], threshold value = 3.

2.4 Pre-processing Video Tracking Data

Intranidal video and feeder images were tracked using the 16-bit BEEtag tracking functions with the settings described above, which generated over 170 million data points across all colonies. This section will first describe potential sources of error that may occur in this raw tracking dataset. Next, this section will outline the steps taken to correct each potential source of error. The aim was to convert this raw tracking data into a format that could be used to accurately describe individual bumblebee behaviour.

2.4.1 Identifying Sources of Error

One unavoidable source of error encountered while implementing a system such as BEEtag for tracking hundreds of individuals was human error. While every effort was taken to mark all individual animals with precision and to keep accurate records, mistakes were rare but did still happen. In most cases, human error leaves an obvious signal or trace in the data that is either easy to identify in analysis of the tracking data after the experiment, if not during the experiment itself.

The other source of error that was present was video tracking errors. These were errors in the sense that the output of the video tracking did not match the true positions of all the tags with full accuracy. Video tracking errors include false tag detections (tags detected in areas of the image where there was no tag, e.g. in the nest substrate), and also missing data (small gaps in trajectories).

2.4.1.1 Tag Identity Errors

Occasionally, two bees from the same colony were accidentally marked with the same tag identity. This situation will cause the identities of the two bees to be indistinguishable, which could confuse the interpretation of individual behaviour from the tracking data. This error can be detected by analysing the tracking data for any occurrences of more than one of the same tag detected in the same image. Doubles of detected tags with the same identity in the same frame can also be caused by tracking errors (see False Tag Detections).

In the situation where a bee's tag fell off during the experiment, there were several possible outcomes. If only one bee was found to be missing a tag and the loose tag was found, the tag was reattached. If the tag was not found, the bee was marked with a new tag and the day was recorded. In this case, the individual bee was linked to the two separate identities that were used to describe it (identifiable by the disappearance of a tag from the dataset on the day that the bee was re-marked, this procedure was only used when there was no doubt as to the original identity of the bee). Finally, if there was more than one bee with a missing tag, there was no way to link their old identities to any new tags. The solutions used to address duplicate tags, missing tags and switched identities will be described in Section 2.4.2.

2.4.1.2 Tag Orientation Errors

Another source of human error was related to the orientation property of tags and occurred when the 'front' of the tag was not aligned with the head of the bee. If the orientation of the tags is a necessary component of the research question then it is going to be crucial to identify instances where this has happened and to correct for it. There were two types of orientation error that were addressed separately: random errors and right-angle errors. The solution to both of these physical tag orientation errors is to 'correct' the digital tag representation of the misaligned tags. This correction involved keeping the digital centroid in place on the centre of the physical tag, while rotating the digital front to align it with the physical anterior-posterior axis of the bee (see Figure 2-9). Rotation correction required the identification of two pieces of information: 1) the identities of the bees with misaligned tags, and 2) the alignment angle by which digital front should be rotated for each individual. When both of these two pieces of information were known, the correction was implemented for every image where the error was present. Details of the frequencies of these errors, the methods to detect and correct them, and the efficacy of the corrections are in the next section (Section 2.4.2).

Right angle orientation errors occur because the tags are square and the encoded front is not clearly identifiable (to the naked eye) within the 16-bit visual code of the tags. Therefore, it was feasible to attach a tag to a bee squared with the anterior-posterior axis, but with the front 'facing' the wrong direction. The result in this case was a set of marked bees where the majority of tags are aligned correctly, but a small minority may deviate from the head direction at 90° intervals.

Random orientation errors, on the other hand, can occur when the researcher has very little or no control over the orientation of the tag (e.g.

when a low viscosity glue is used to attach tags) and as a result the orientation could deviate from the front by some continuous angle. Instances of random orientation errors in the present study were attributable to low-viscosity glue, which was unsuitable for fine control of the tag orientation during the marking process.

2.4.1.3 False Tag Detections

False tag detections occur when the `locateCodes` function detects the visual pattern of a tag in an area of an image where there is no tag (e.g. in shadows on the nest substrate). These false tag detections were relatively rare, but could have serious effects on the interpretation of behaviour from tracking data because the spatiotemporal attributes of false detections would be associated to the identities of the bees marked with the falsely detected tag. For example, measuring the movement speed of individual bees from the raw tracking data (without correcting for these errors) produced extreme outliers with high average speed measurements in some colonies (see Figure 2-7). Impossible speed measurements were generated when a single false detection occurred as part of an otherwise continuous sequence of real tag detections, but was detected in a distant part of the nest relative to the real tag. This random occurrence can lead to the calculation of impossibly large measurements of the distance travelled between successive frames and ultimately affect the accuracy of automatically generated behavioural data.

The majority of cases of false tag detections involve only a small subset of tag identities (out of the 328 tags used). It is clear that the tag identities most commonly involved in this kind of error have a visually simple pattern of pixels in the encoded binary matrix (Figure 2-6). The simplest approach to

avoiding these errors in future studies would be to blacklist any tags that commonly generate errors. For the present study, several techniques were developed to remove false detections from raw tracking data. These techniques and their efficacy will be discussed below.

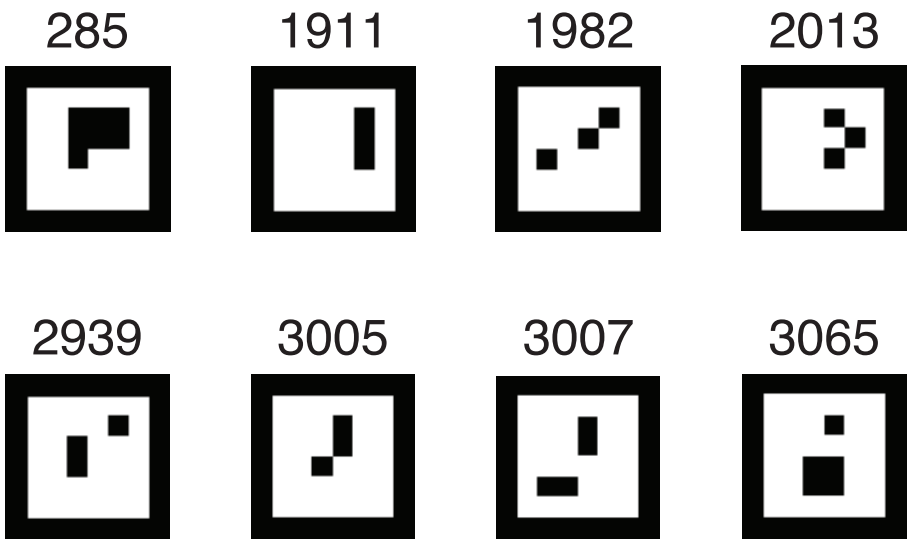

Figure 2-6. Visually simple, error-prone tags. The top eight tags most likely to be falsely detected in image regions where there is no physical tag present (ordered by tag number).

2.4.1.4 Missing Data

When a bee's tag was visible to the camera, the sequence of detected tags through time was often made up of many short continuous trajectories, broken up by frequent missing frames. Even if a tag was visible in an image or video to the naked eye, the tracking process was imperfect and did not always detect the tag under real experimental conditions. These missing frames can also be caused by motion blur or brief occlusions of the tag by the nest structure or other bees. In order to improve the continuity of individual movement trajectories, missing frames were 'filled in' by interpolation of missing data points. However, some gaps in the trajectories of individual bee represent significant shifts in behaviour such, as leaving the nest or retiring from view beneath the brood comb. The duration of the gaps in trajectories caused by individual behavioural shifts were typically longer than tracking errors and this distinction was investigated in the next section.

2.4.2 Correcting Errors

The pre-processing steps described in the following subsections are presented in the order in which they were applied to the raw tracking data. These techniques were developed to address sources of error in the tracking data generated from nest videos, therefore, all examples and results will be in reference to the nest video tracking data. However, many of the solutions are generic and some were also applied to the tracking data generated from feeder photos (the steps 'maximum distance threshold' and 'removing singles' were not applied to forage photo tracking data.).

2.4.2.1 Tag Blacklist

The first step in pre-processing was to completely remove tags causing significant errors that could not be corrected. This step was considered a last resort, when there were no other options left to correct an error made during data collection. Blacklist errors included: double tag identities, and uncertain tag identities after loss of tags from more than one bee. The method to identify double tag identities from the tracking data is described in the next section. Removing blacklisted tags was implemented as a MATLAB function.

2.4.2.2 Removing Doubles

In most cases, two different bees with the same tag were noticed during the experiment, in which case, the single common tag identity was recorded, added to the blacklist, and removed from the dataset. The tracking data was also analysed to detect the presence of double tag identities in case any had been missed. A simple MATLAB function was developed to record the presence of any tag identity that was detected twice in the same frame.

The number of doubles detected per tag in Colony J on day 13 is given as an example. Out of the 72 unique tag identities that were detected in the colony on that day, 32 tag identities were detected twice in the same frame at least once. Of those 32 double tag identities, 24 were only detected as a double in a single frame (out of 90,000 frames in an hour), 7 were detected as a double in the range of 2-18 frames, while the final tag (#596) was detected as a double in 532 frames. This frequency of doubles was still low compared to the total 90,000 frames in one hour; however, it was at least an order of magnitude greater than for any other identity. This suggests that more than one bee with the same tag may have caused this relatively high frequency of doubles. This qualitative assessment was confirmed visually by overlaying the

tracking results of code #596 on video frames where the double was present, which showed that there were two bees marked with the tag #596. This process of examining the frequencies of doubles followed by checking the overlaid tracking data was carried out for each video. During the course of marking 1168 bees across 10 colonies for this study, there were only four double tag identities caused by human error (eight bees in total). As there was no way to distinguish between bees with the same tag, the identities were added to the blacklist and they were removed completely from the dataset to avoid mixed identities.

The simplest way to address the remaining doubles caused by tracking errors was to remove any doubles from the frame in which they occurred. Doubles caused by tracking errors were made up of a real tag detection and a false tag detection in the same frame. Ideally, only the false tag detection would be removed, but distinguishing the real tag from the false tag may not always be possible without manual confirmation; therefore, the simpler option was to remove both. The loss of one real tag in exchange for successfully removing a false tag was justifiable for several reasons. The number of doubles removed across the entire dataset was 29,209 (out of 177,509,598 detected tags; see Figure 2-17). This includes both real and false tags; therefore, the percentage of real tag detections lost was $<0.0016\%$. Additionally, correcting for missing data was achieved by interpolation (see Section 2.5.2.5), which can easily account for occasional missing frames of real trajectories that were remove by this step.

2.4.2.3 Maximum Distance per Frame

The distance per frame (DpF) measurements used to calculate individual movement speeds were also used to detect and remove false tag detection

errors. As described above, when a false tag detection occurs in temporal sequence with a trajectory of real tag detections, it can occasionally generate very large DpF measurements when it is also distant from the real trajectory. These temporally adjacent and spatially distant tag errors were addressed by removing any tags in adjacent frames that were more distant than a maximum DpF threshold. As with the removal of doubles caused by false tag detections, this method removes a real tag detection for every false tag detection, which is justifiable for the same reasons, namely the number of real tags removed was removed was small and interpolation can correct for some of these 'real' missing data (see Figure 2-7).

The maximum DpF threshold was determined by investigating the pairs of tag detections that generated the largest DpF measurements in Colony J on day 5 (Figure 2-7). The tag detections that generated these largest DpF measurements were each overlaid on the source video frame and the status was scored as either a true DpF measurement (two consecutive true tag detections) or a false DpF measurement (one true and one false tag detection, or two consecutive false tag detections). There were no false DpF measurements caused by two consecutive false tag detections. The largest true DpF measurement in this dataset was 96 pixels; all larger outliers were false DpF measurements. Based on this assessment, a threshold value of 100 pixels per frames would successfully remove tag detection errors in this dataset. The DpF measurements of the trajectories of all bees across all colonies are shown in the histogram in Figure 2-8. It is clear from this figure that DpF measurement $>$100 pixels were extremely rare; therefore this threshold was applied to all colony datasets. Indeed, this processing stage only removed 7,298 tag detections overall, which was 0.004% of the size of the data set after removing the black list and the doubles (see Figure 2-17). Any

remaining false tag detections that occur adjacent to real trajectories will be within 100 pixels, which is within the possible range of movement an individual bumblebee.

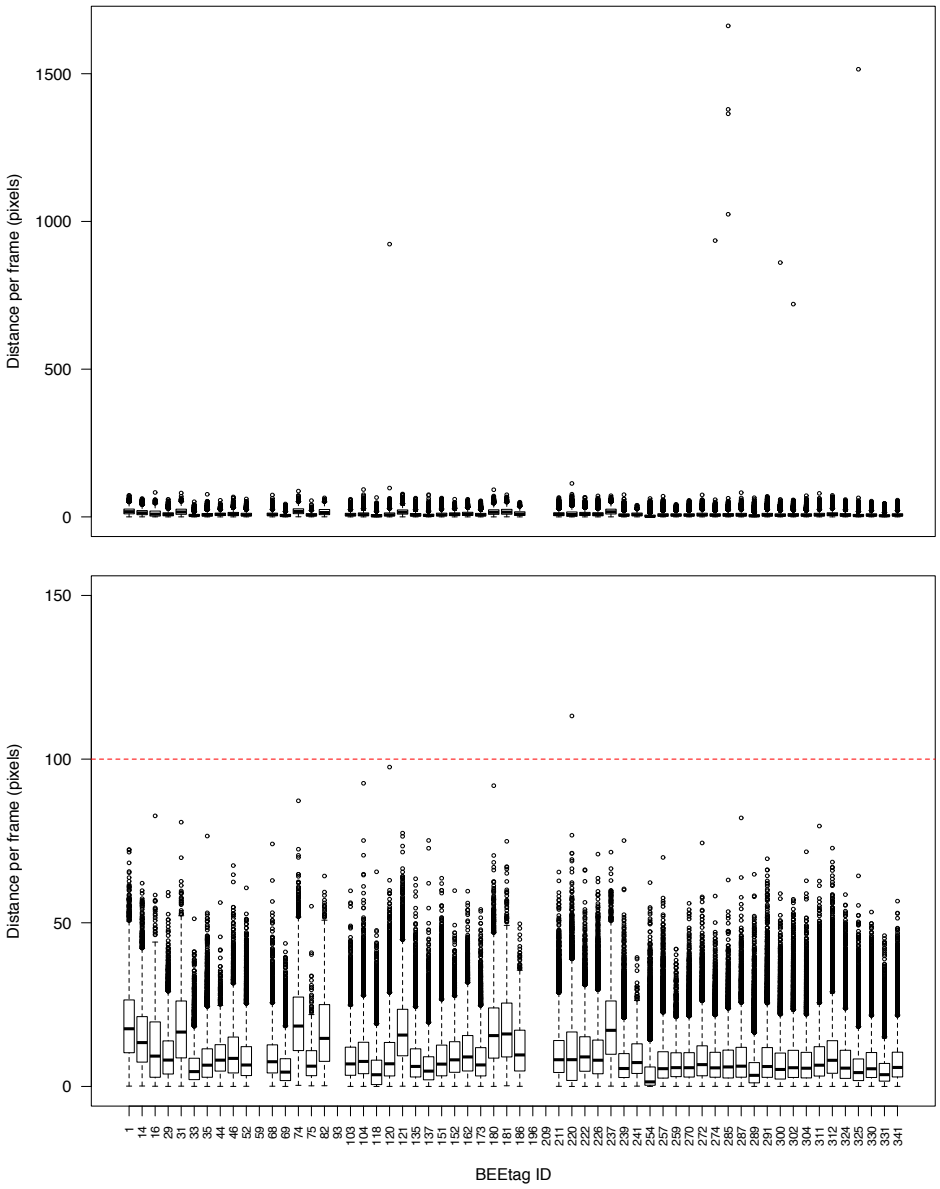

Figure 2-7. Distance per frame outliers. Distribution of distance per frame (DpF) measurements for each individual in Colony J on day 5. The top panel shows the full range of DpF values. The bottom panel shows the same DpF measurements, but with the y-axis scaled from 0-150 pixels. The dotted red line in the bottom panel shows the maximum DpF threshold (100 pixels).

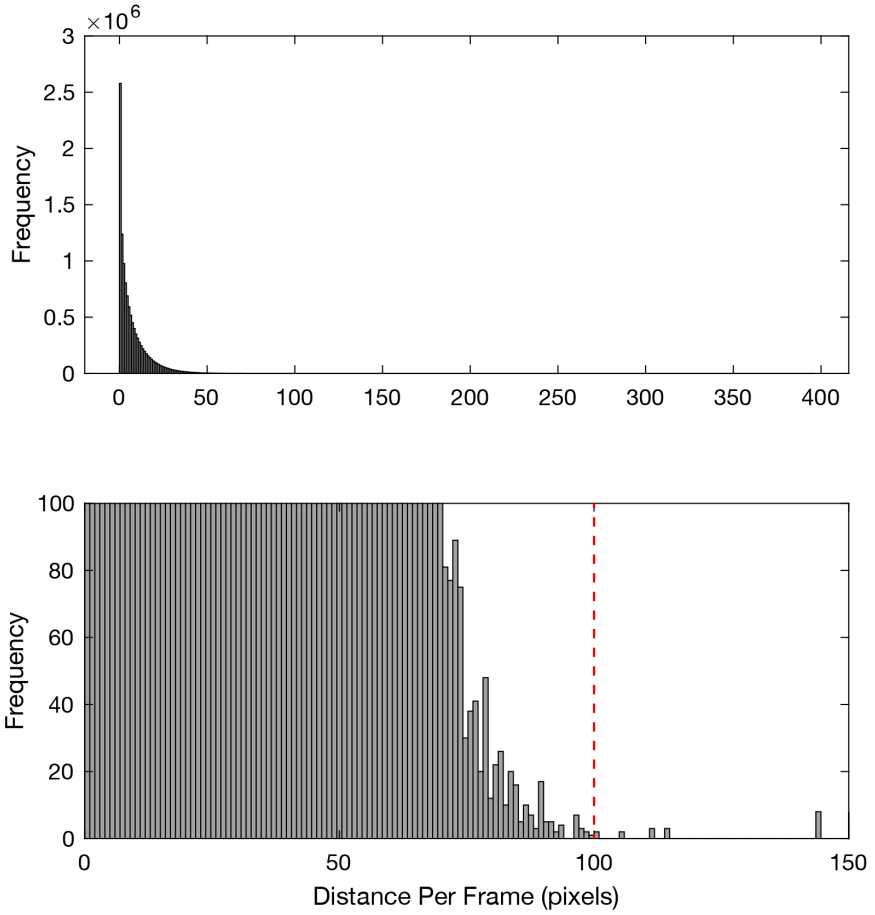

Figure 2-8. Distance per frame histogram. Distribution of distance per frame (DpF) measurements for all colonies and all days. The top panel shows the full distribution, while the bottom panel shows both the x-axis and the y-axis re-scaled. The dotted red line in the bottom panel shows the maximum distance per frame threshold (100 pixels).

2.4.2.4 Tag Orientation Correction

The method for correction tag orientation errors was based on identifying when the encoded front of the tag did not align with the anterior-posterior axis of the bee, calculating the angle of deviation between the encoded front and the head, and finally rotating the encoded front so that it would align to the head. This angle of deviation was equivalent to the angle of *drift* in aeronautical navigation terms, which describes the difference between the *heading* vector (the direction the vehicle is pointing) and the *course* vector (the direction in which the vehicle is actually travelling). One way to calculate the drift angle of tags was to take the vector between the centroid **C** and the front of the tag **F** as the heading vector $\overrightarrow{CF}$, and the vector between **C** and the head of the bee **H** as the course vector $\overrightarrow{CH}$ (Figure 2-9). The `atan2` function in MATLAB was used to calculate the polar angle of $\overrightarrow{CF}$ (ϑ_1) and $\overrightarrow{CH}$ (ϑ_2), with respect to **C** as the origin (Figure 2-9). The difference between ϑ_1 and ϑ_2 returns an angle in degrees in the range [−360, 360], which does not map to a full circle, therefore the final drift angle ϑ was equal to (ϑ_2− ϑ_1) modulo 360. The result was an angle in the range [0, 360] degrees that described the angle between the orientation of the tag and the head of the bee in polar coordinates (see Figure 2-9). The drift angle ϑ was used to correct the error by rotating the misaligned $\overrightarrow{CF}$ about the centroid by 360°-ϑ, with respect to the centroid at the origin, which aligns $\overrightarrow{CF}$ with $\overrightarrow{CH}$. This rotation according to the correction angle was applied to every frame where a misaligned tag was detected. The identification of misaligned tags and the calculation of the drift angle were slightly different for random orientation errors and right-angle orientation errors and will be described below.

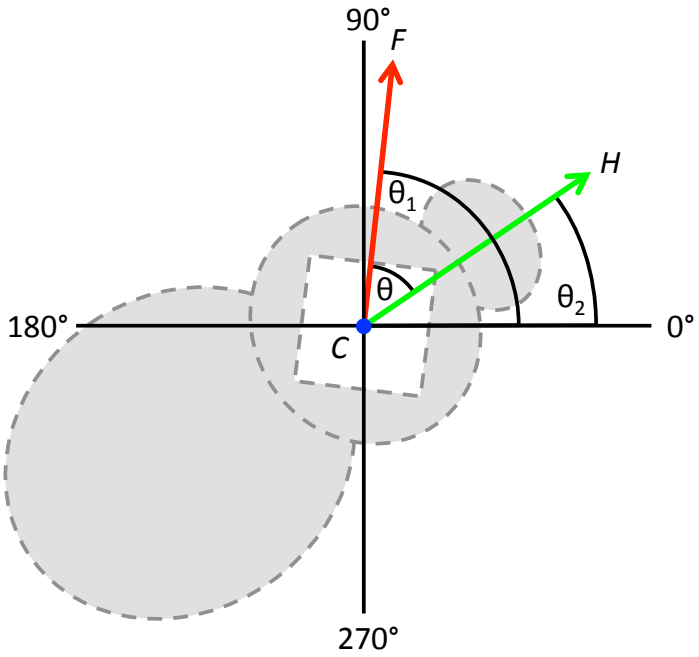

Figure 2-9. Tag orientation error diagram. Diagram of a bumblebee (gray dashed ellipses) marked with a misaligned tag (dashed white square). The head of the bee is directed toward the point **H**, while the encoded front of the tag is directed toward the point **F**. The centroid of the tag is shown as the point **C**. The tag orientation must be corrected by 360° minus the angle ϑ, referred to as the drift angle. The drift angle ϑ is calculated as $(\vartheta_2 - \vartheta_1)$ modulo 360, where ϑ_1 and ϑ_2 are the polar angles of the vectors CF and CH, respectively.

Identifying individuals with random angle tag orientation errors was straightforward because the tags were square and the encoded front of the tag was located along the middle of one side of the square; therefore, it was immediately apparent when a tag was misaligned with respect to the anterior-posterior axis of the bee. The identities of the individuals with these random errors were recorded as soon as the tag was incorrectly attached to the bee. In general this was only necessary for Colony F and Colony G because the low viscosity glue used at the time to attach the tags gave no alignment precision, which resulted in essentially random tag alignment in these two colonies. To correct these errors, the drift angle of bees with random tag orientation errors was calculated precisely for each individual. This procedure required images of individual bees with the tag and head clearly visible. In the case of Colony F and Colony G each bee that required a custom orientation correction was photographed individually post mortem. Images could also be acquired as frame grabs from the colony video as necessary. A simple MATLAB app was built to load an image, detect the tag in the image (using the `locateCodes` function), and ask for the user to input the position of the head of the bee by positioning the cursor with the mouse (Figure 2-10). The drift angle was calculated as above and the correction angle was saved for the specific individual.

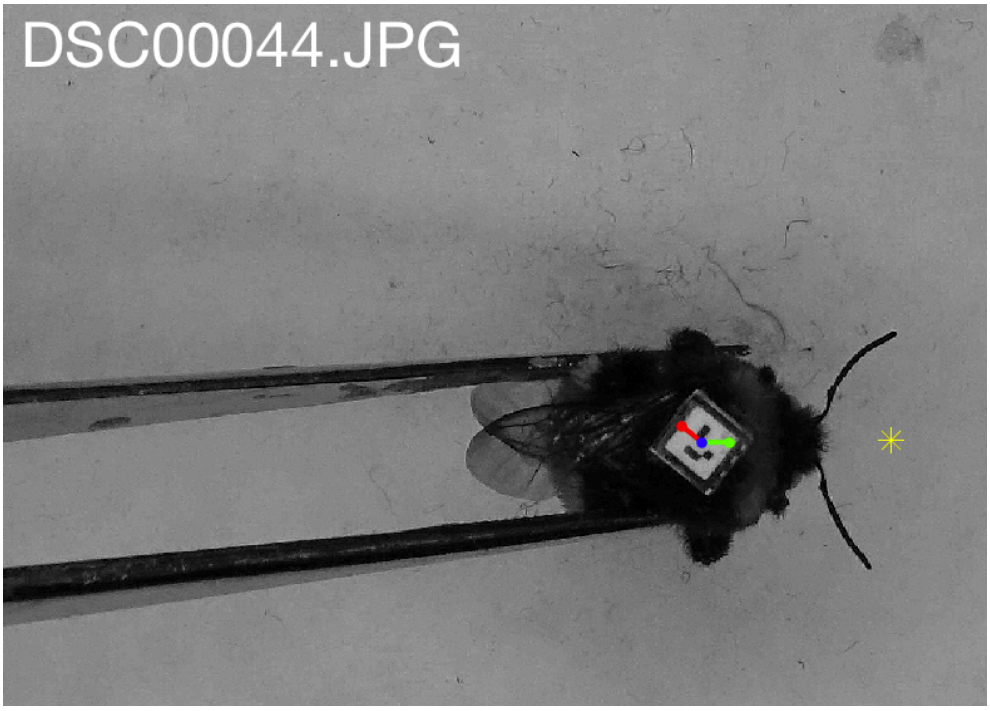

Figure 2-10. Random angle tag orientation error labeller app. Screenshot from the MATLAB app designed to calculate the tag alignment correction angle from images of individual bees. The results of the detected BEEtag are plotted on the associated tag in the image: the blue point is the centroid of the tag; the red point is the front of the tag. The yellow star is the head orientation inputted by the user. Given these inputs the app calculates the angle between the centroid-front vector and the centroid-head vector then rotates the front point about the centroid and plots the new front orientation (the green point). Text displays the image file name.

Right angle tag orientation errors were less obvious to identify because the tag was correctly aligned with the anterior-posterior axis, but was 'facing' in the wrong direction. This identification problem led to the development of a custom MATLAB script to analyse the raw tracking data and calculate drift angles without defining the head of each individual bee. In this case the heading was the same $\overrightarrow{CF}$ vector as above but the course vector used for the calculation was the movement vector of the bee between successive frames (Figure 2-11). The points of a centroid at time t ($\mathbf{C_t}$) and at time t+1 ($\mathbf{C_{t+1}}$) described the movement vector $\overrightarrow{C_tC_{t+1}}$ of an individual bee between successive frames. Assuming bees walk forwards the majority of the time, and there are many successive frames to sample from, the movement vector $\overrightarrow{C_tC_{t+1}}$ should act as a proxy for the exact head vector $\overrightarrow{CH}$ and can be measured automatically for every bee present in the tracking data. If a bee's heading was north and its course was also north, the drift angle would be zero. Equivalently, if a bee moves in the same direction as the front of the tag is facing, the drift angle would also be zero. Thus, if a bee's course is north, but the tag front heading is east, the drift angle would be 90°, which represents a right angle tag orientation error. The result of the script was $N - M$ drift angle measurements for each individual, where N was the sum of the frames that were part of trajectories, and M was the number of separate trajectories.

This procedure for identifying and correcting right angle tag orientation errors was not entirely automated; the next step required user input with the help a heat map visualisation. A heat map of drift angle measurements for multiple individuals enables rapid identification of tag orientation errors by eye because of the grouping of correlated measurements (**see** Figure 2-12). To generate heat maps of alignment angles, the frequencies of binned polar angle results for each bee were normalised by bee and arranged by hierarchical

clustering. The result of the clustering was shown with an associated dendrogram that tended to cluster correctly aligned tags and tag alignment errors separately. The angle that a particular tag needed to be corrected by was read by eye from the heat map and saved to a reference file.

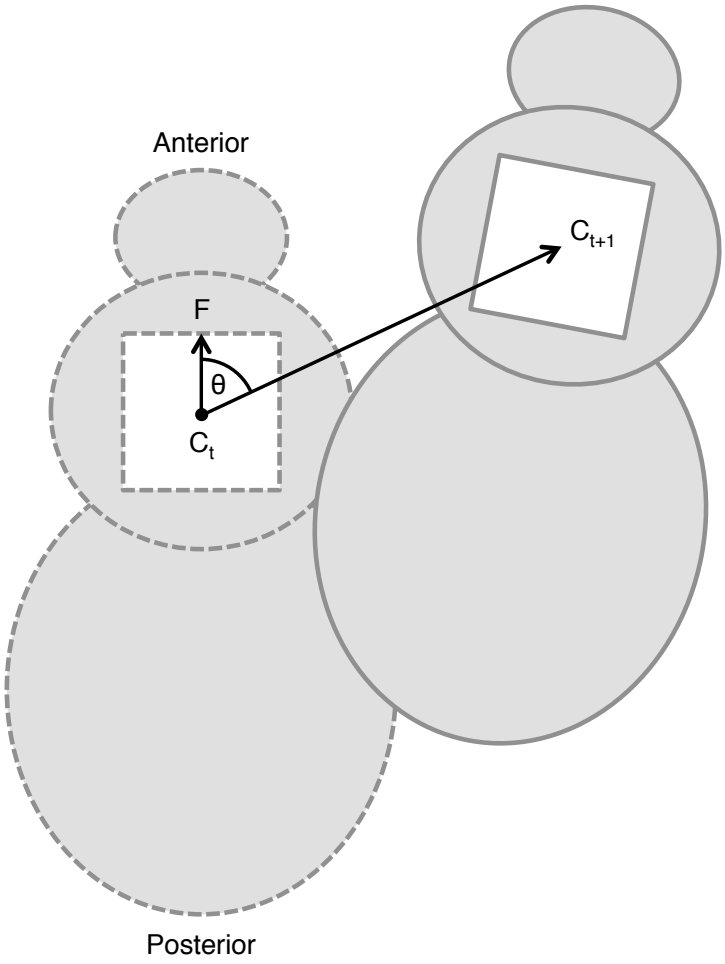

Figure 2-11 Calculating drift angle from trajectories to identify right angle tag orientation errors. Diagram of the drift angle (ϑ) measured to distinguish correctly aligned tags from misaligned tags. The dashed ellipses represent the body of a bee at time *t*. The solid ellipses represent the same bee at time $t + 1$. The white squares represent the tag attached to the bee. The point **F** is the front of the tag at time *t*; $\mathbf{C_t}$ is the centre of the tag at time t; $\mathbf{C_{t+1}}$ is the centre of the tag at time $t + 1$;. The time step t is 0.04s, the time interval between video frames.

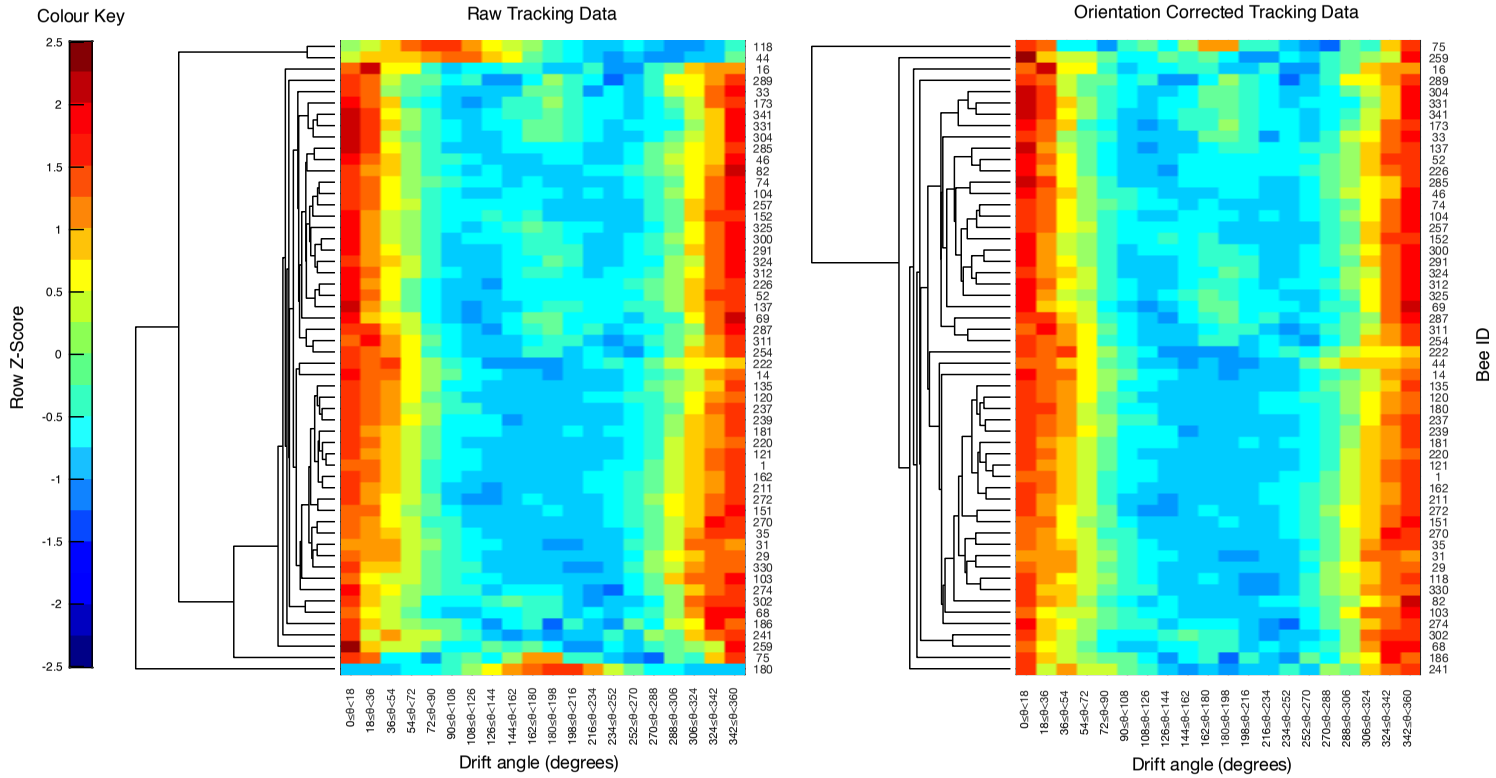

Figure 2-12. Right angle tag orientation correction heat map. Heat map of drift angles measurements in the range [0, 360] degrees representing the degree to which the movement vectors of tracked bumblebees differ from the front orientation of their video tracking tag. The rows show individual bee IDs (from Colony J, day 5) and the columns show binned drift angle frequencies. Angle frequencies are shown as Z-scores normalised by row. When a bee has positive Z-scores near 0° and 360°, this represents high frequencies of measurements where the tag front and the movement vector have a similar orientation. High Z-scores at 180° represent measurements where the tag front and the movement vector have opposite orientations. Dendrograms show grouping of similar rows by hierarchical clustering. The left panel shows drift angles calculated from raw tracking data. This shows most bees walk in the direction the tag is facing. Tag #118, #44 and #180 show right angle errors. The panel on the right shows the same tracking data, but with these errors corrected by rotating the encoded tag fronts.

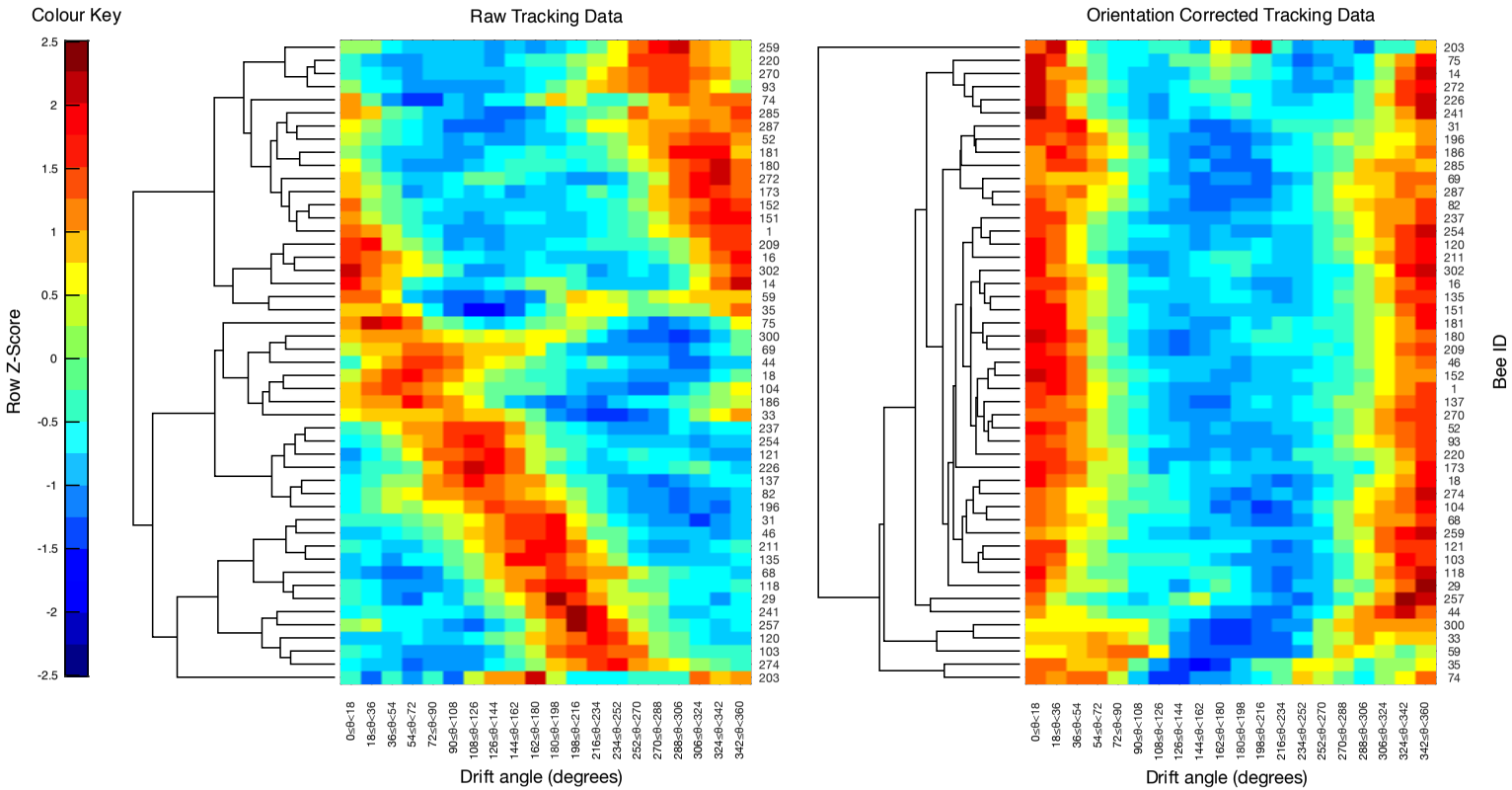

Figure 2-13. Random angle tag orientation correction heat map. Heat map of drift angles measurements in the range [0, 360] degrees representing the degree to which the movement vectors of tracked bumblebees differ from the front orientation of their video tracking tag. The rows show Individual bee IDs (from Colony F, Day 1) and the columns show binned drift angle frequencies. Angle frequencies are shown as Z-scores normalised by row. When a bee has positive Z-scores near 0° and 360°, this represents high frequencies of measurements where the tag front and the movement vector have a similar orientation. High Z-scores at 180° represent measurements where the tag front and the movement vector have opposite orientations. Dendrograms show grouping of similar rows by hierarchical clustering. The left panel shows drift angles calculated from raw tracking data. Given bees tend to walk forward (Figure 2-12), this shows the tags of most of these bee not aligned with the head. The panel on the right shows the results of the corrected tag orientations.

The raw tracking data from Colony J on day 5 was used as an example of the right angle tag orientation correction process. The results of the calculation of drift angles from the raw tracking data show that bumblebees tend to walk in the direction of the front orientation of their tag, which was directed towards the head (Figure 2-12). In other words, bees walk forwards. Given that this assumption was true for most bees, the heat map clustered several bees that did not fit the pattern: #118, #44, #180, and #75. The frequency of drift angles for #118 and #44 show that the movement vectors of these bees tend to produce drift angles relative to the front of their tags of ~90°. This result suggests that the front of these tags were oriented towards the left of these bees, which was confirmed by checking the bees' tags post mortem. Bee #180 had a high frequency of drift angles at ~180°, checking the tag on the bee post mortem confirmed that the tag was on backwards. Finally, #75 shows a high frequency of drift angles at ~0/360° and at ~180°, which suggests the bee moves both forwards and backwards with relatively high frequency. Once again, the bee's tag was checked post mortem, which showed that the tag was on straight and did not need correction. For the bees with tag orientation errors (#118, #44 & #180), the correction angle was saved and the correction was applied across all videos. Figure 2-12 also displays the results of the correction, which shows that this procedure can be used to confirm the correct tag orientation of an entire colony at a glance and that the correction can be confirmed with a high level of certainty.

The drift angle heat map procedure was also used to confirm the results of the correction angles applied to tags with random orientation errors. Figure 2-13 shows the drift angle frequencies of the raw tracking data in Colony F on day 1, which confirms the random distribution of tag alignment errors in the

colony. The results of the correction show that this procedure for correcting random tag orientation error was also highly effective.

2.4.2.5 Interpolating Missing Data

Missing data points in trajectories can be corrected by constructing new data points within a window of existing trajectory data points in a process called interpolation. This method can be used to construct new, evenly spaced data points along a straight line where there are gaps in trajectories. Linear interpolation generates accurate approximations of missing data when the distances between existing data points are small. However, increasing the window size between known data points will decrease the accuracy of the interpolated data, as the interpolation could smooth over local underlying signals in the original data. With a focus to reduce the number of gaps in video tracking trajectories, while avoiding generating inaccurate data points, it was important to identify an appropriate maximum window size.

The maximum window size for interpolation was set based on an analysis of the typical size of trajectory gaps in terms of time and distance. The tracking data from Colony J day 5 was used as a representative example of the analysis of missing data. This example data was processed according to all of the previously described steps before being used here. In the 1-hour of video tracking data on this day, there were 141,104 gaps in individual trajectories, ranging in duration from 1 frame (1 video frame at 25 frames per second = 0.04 seconds) to 41,812 frames (~28 minutes; Figure 2-14). According to Figure 2-14**,** a maximum interpolation frame window of 1 frame would 'fill in' close to half of all gaps (46.2%). Increasing the duration of the maximum frame window beyond 1 would have diminishing returns in terms of the number of gaps filled in. The maximum frame window used for

interpolation was 8 frames. This frame window corresponds to a relatively conservative 0.32 seconds, but corrected 83% of all trajectory gaps in the example dataset. Additionally, the maximum distance over which data points could be interpolated was ~42mm (approximately three bumblebee body lengths). In reality the interpolation distance was much smaller. The mean distance between individual trajectories separated by a gap of 8 frames was 3.18 mm, which was shorter than the average thorax width in this colony (Figure 2-15).

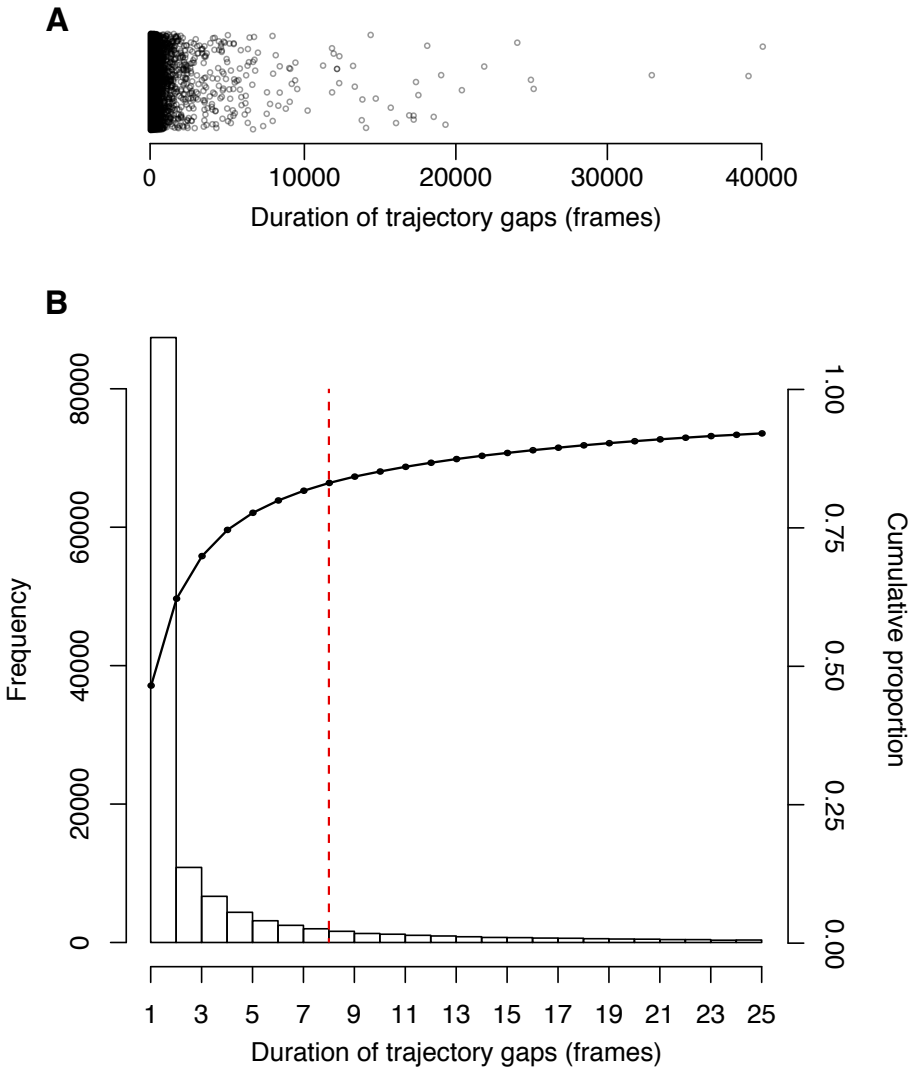

Figure 2-14. Durations of gaps in trajectories. Panel A shows the duration of all gaps in trajectories from the processed tracking data of Colony J on day 5. Points are distributed randomly on the y-axis. Units are video frames recorded at 25 frames per second (1 frame = 0.04s). Panel B shows a histogram of the frequencies of the same gaps in trajectory data as above, but with the x-axis re-scaled to only show gaps up to ~1 second long. Panel B is also overlaid with a cumulative frequency curve (shown as the cumulative proportion of all observations). Dotted red line shows the maximum gap duration over which to interpolate missing data points (maximum window size = 8 frames).

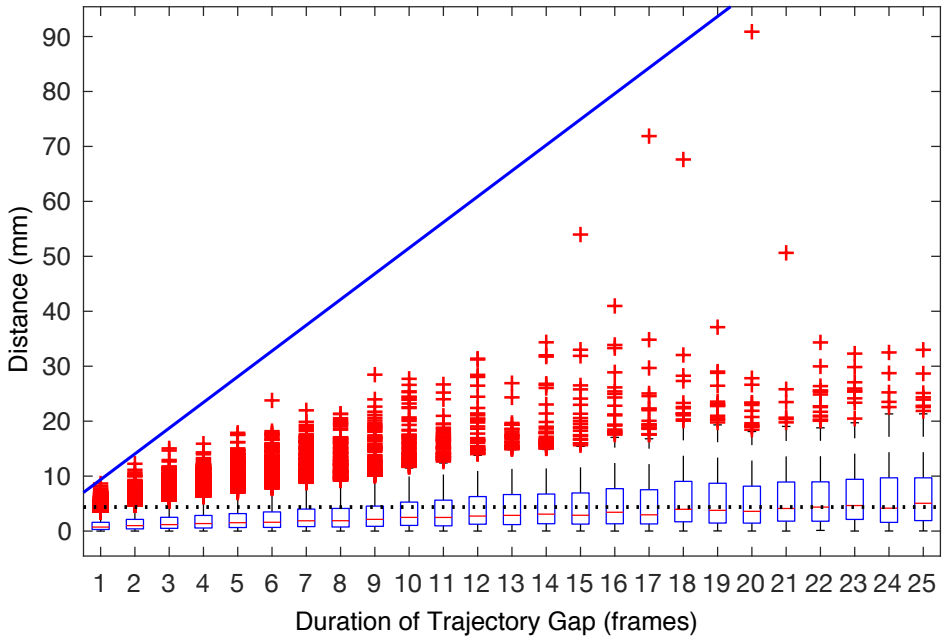

Figure 2-15. Distances of gaps in trajectories. The distance between the start and end of every gap in individual trajectories plotted against the duration of the gap. Results are from the tracking data of Colony J, day 5. The solid blue line shows the maximum possible distance for each gap duration imposed by the maximum distance per frame limit (see Section 2.4.2). The dotted black line represents the mean thorax width of bumblebees in this colony. The x-axis units are video frames recorded at 25 frames per second (1 frame = 0.04s)

Before the interpolation procedure was implemented on all tracking data, the potential impact of any remaining false tag detection errors was assessed. Although the maximum distance per frame threshold removed errors connected to true trajectories by large distances, interpolated data points would connect any false tag detection within 8 frames of a real trajectory. A new maximum interpolation distance could account for this specific case, but given that thousands of false tag detections connected to trajectories had already been removed, it was highly likely that there were many more undetected temporally isolated 'single' false tag detections (i.e. trajectories of length 1) not connected to any continuous trajectories. Removing all of these single detections, following all other previously described error-processing steps, could therefore delete many errors that had not previously been detected. However, these single detections were not just errors, they also made up disjointed parts of real trajectories. For example, a raw trajectory could be made up of alternating single detections and missing frames. Removing the single detected tags from this example would delete the entire trajectory. An alternative would be to interpolate first and then remove single tag detections. The effect of the order of these two processes could therefore have a significant effect on the outcome of this stage pre-processing.

The two opposing process of interpolation and removing single detections could be considered equivalent to the morphological image processing operations of *dilation* and *erosion*, respectively (Soille, 2004). When processing a binary image (a matrix of 1s and 0s), dilation expands any connected sets of 1s in the image, while erosion shrinks any connected sets of 1s. Additionally, a shape called a structuring element sets the 'rules' of the dilation and erosion operations, which affects the shape of the outcome. In a similar way, interpolation expands connected sets of tag detections (given the

rule that trajectories can expand if there is a single tag within 8 frames) and removing single observations shrinks trajectories (given the length of the trajectory is 1). The two different sequences that erosion and dilation can be applied in are considered as separate compound operations in morphology called *opening* (erosion followed by dilation) and *closing* (dilation followed by closing), which have different effects on the final image (see Figure 2-16).

Opening and closing, in terms of processing tracking data, had different effects on the final dataset. The total number of tag detections from the entire dataset (178 hours of video) after all previously described pre-processing steps had been applied was 177,473,010 detected tags (Figure 2-17). During the opening operation, removing singles first deleted 72,252 detected tags, and then interpolation (over an 8-frame window) generated 40,212,597 data points, resulting a final dataset of in 217,613,355 data points. Alternatively, during the closing operation interpolation first generated 40,328,024 data points, and then removing singles deleted 8,302 detected tags, resulting in a final dataset of 217,792,732 data points. Ultimately, the difference in the number of data points (179,377) was nearly negligible relative to the quantity of tags in either final dataset. The effect of these two compound operations on the *quality* of the tracking data may not be measurable without manually scoring the number of positive tag detections; therefore, the intended effect of each was considered. In morphology, opening is used for removing small features, trimming small protrusions from the edges of features, and for breaking narrow isthmuses connecting features. On the other hand, closing is used for removing small holes in features, expanding small features and fusing small breaks (Soille, 2004). On this basis, opening was the preferred processing operation because it had a greater chance of

removing small single errors and a smaller chance of connecting real trajectories to single errors within the interpolation window.

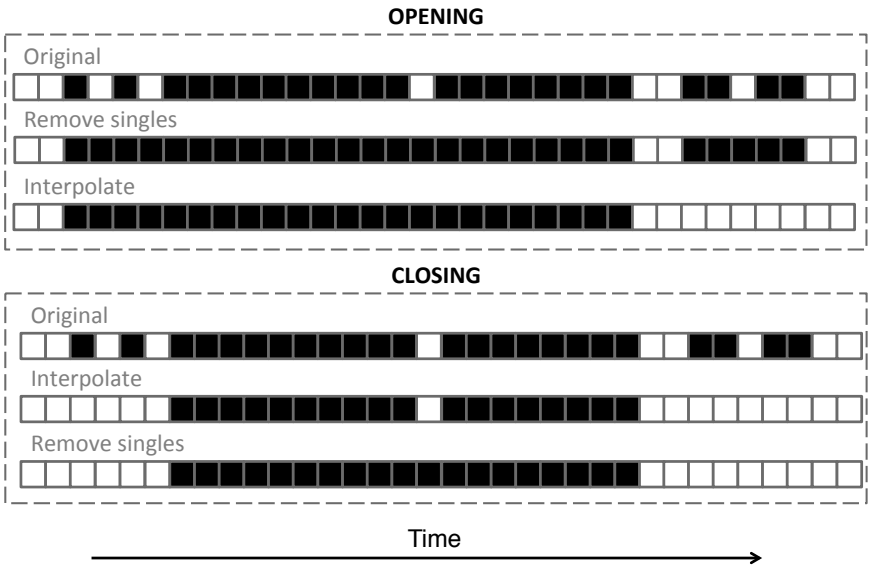

Figure 2-16. Opening and closing operations for missing data. Visual representation of the potential for different outcomes of removing single observations first then interpolation over an 8-frame window ('opening') versus interpolating first then removing singles ('closing'). Rows of squares represent video tracking trajectories moving through time from left to right. Each square represents a video frame, white frames contain a tag detection, black frames have no tag detection.

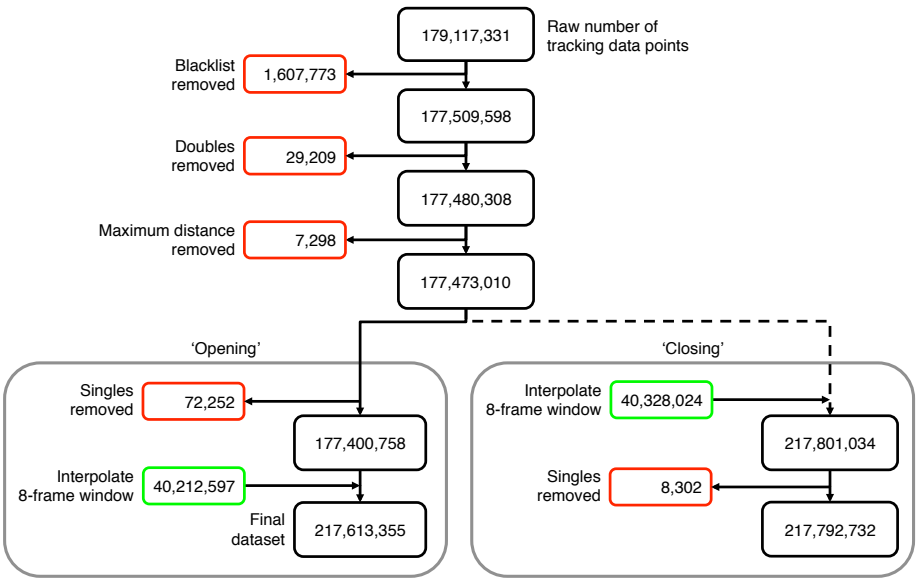

Figure 2-17. Tracking data pre-processing flow chart. Flow chart following the effect of each stage of pre-processing on the nest video tracking data (in terms of the number of data points of all bees across in all ten colonies across all 178 hours of video). Red boxes show where pre-processing stages deleted data points and the number that was deleted. Green boxes show where stages generated new data points and the number that was generated. The compound stage 'opening' was used to process the final dataset, but alternative compound process 'closing' is shown for comparison.

2.5 Interpreting Behaviour from Video Tracking Data

2.5.1 Locomotor Behaviour

Individual activity was recorded as the median instantaneous speed over each 1-hour monitoring period. Instantaneous movement speed was calculated from the video-tracking data by taking the distance travelled between consecutive video frames, divided by the video frame interval time (0.04 seconds per frame). The median movement speed was used as opposed to an absolute measurement of activity such as total distance, which has been used in previous studies employing video tracking to record bee activity (Alkassab and Kirchner, 2018; Charreton et al., 2015; Teeters et al., 2012). It was not possible to measure total distance travelled because the BEEtag markers on bees in queenright colonies are not visible at all times. This means there are gaps in the video-tracking data when markers are out of view (e.g. they can be obstructed other bees or by the brood comb). Movement speed results are presented in Chapter 3.

Two different metrics of spatial distribution inside the nest were used to describe individual bees. The first metric was based on the social centroid of the nest, which was estimated by taking the mean x-y coordinates of all bees inside the nest over each 1-hour observation period. The video tracking data was used to calculate the instantaneous distance from the social centre of each bee in each video frame (every 0.04s) it was present. The median distance from the social centroid was used as a single metric of spatial centrality per individual per day (Sendova-Franks and Franks, 1994). The second metric of space use was "home range" inside the nest, and was recorded by estimating the area of the minimum convex polygon that describes 50% of a bee's spatial positions (see Crall et al., 2018). This home

range metric was also measured per individual per day. Space-use results are presented in Chapter 3.

2.5.2 Foraging Behaviour

Foraging behaviour was quantified automatically from patterns in the tracking data of individual bees as they moved between the nest and the feeder. In order to follow individuals between the nest and the feeder, the tracking data from both locations was synchronised. The 1-hour nest video defined the start time and the end time of sampling on each day. The start time was read from the metadata of each video using the free software ffmpeg (https://www.ffmpeg.org/about.html) and saved as a json file. The end time was taken as exactly one hour after the start time. A vector of time values was generated from the video start time to the video end time, at the maximum frame rate of the feeder camera (2 fps). This vector of 7,200 time values was used to match the sporadic feeder image tracking to the reference time set by the nest video. Any tracking data outside of the start and end time of the nest video was deleted. The result was two tracking data streams synchronised at different frame rates.

Interpreting foraging behaviour from synchronised nest and feeder data steams was required a definition of a foraging bout that could be accurately recognised from video tracking data. A complete foraging bout was defined as a round trip from leaving the nest, to arriving at the feeder, and back again to the nest. This round trip was simplified to the sequence of observations: 1) present in the nest, 2) present in the feeder, 3) present in the nest; which is a signal that can be easily read from the data. The implementation of this approach considered every single bee to be in one of three states at any one time: *nest*, *absent*, or *feeder*. A bee was in the *nest* state when its tag was

detected in video frames from the nest camera. A bee was in the *feeder* state when its tag was detected in images from the feeder camera. The *absent* state occurred at times when the bee was not detected in the visual data from either camera. It was not possible for a bee to move directly from the *nest* state to the *feeder* state without moving out of view of both cameras and passing through the *absent* state. Therefore, expanding on the sequence described above, the new foraging bout sequence can be represented as *nest-absent-feeder-absent-nest*.

In practice, the sequence of states of each individual was saved as a character vector with one of three letters corresponding to each state: tag present in nest *N*, tag absent *A*, tag present at feeder *F* (Figure 2-18). This state vector was recorded at the frequency of the nest tracking (25 fps). The feeder tracking state vector (2 fps) was resampled by nearest neighbour interpolation to generate a vector of length 90,000 to match the length of the nest state vector before they were combined into the final 3-state vector. Foraging bouts were detected by regular expression analysis of the individual state character vectors. The verbose rules for the regular expression analysis can be described as: three *N*s, followed by one or more consecutive *A*s, then followed by at least one *F* plus any number of other letters until the next nearest three *N*s (Figure 2-18). The three *N*s at the beginning and end of the bout ensure that the bee was definitely inside the nest and not caused by an undetected tracking error. The number of *A*s and *F*s were not fixed to account for foraging bouts of variable length. The outcome of this analysis was a start time and an end time of every round-trip foraging bout of every individual. Foraging results are presented in Chapter 3, and forager classifications in Chapter 4 are based on these data.

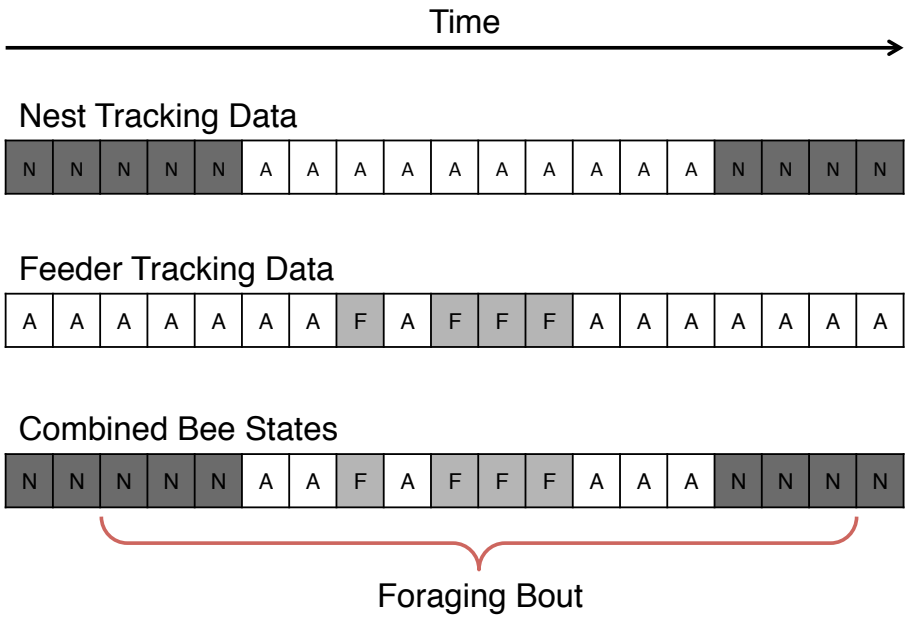

Figure 2-18. Foraging bout detection. Sequences of states derived from the presence and absence of an individual bee's tag in the tracking output of the nest video and the feeder photos. Boxes represent specific time points. Absence is represented by an 'A', presence in the nest is 'N', and presence at the feeder is 'F'. A foraging bout is defined by the sequence shown in the combined bee states.

2.5.3 Social Interactions

Video tracking data generated via the BEEtag system was used to automatically record social interactions between pairs of bumblebees inside the nest according to two different detection techniques: proximity interactions and 'head-to-head' interactions. The results of tracking social interactions are presented in Chapter 4.

2.5.3.1 Proximity Interactions

The first technique aimed to detect physical contact interactions that occur between pairs of bees inside the nest. Contact interactions are known to influence individual foraging behaviour (Renner and Nieh, 2008) and can transmit disease (Otterstatter and Thomson, 2007) in bumblebees. Physical proximity was used to approximate direct contact between pairs of bees, thus the interactions detected via this technique will be referred to as proximity interactions. Proximity interactions were recorded when the movement trajectories of individual bees were within 10mm of each other. This minimum distance was set to ensure that pairs of bees would be close enough for direct physical contact. This method does not account for individuals of different body sizes, but Otterstatter and Thomson (2007) found that across a range of threshold distances (6-15mm) there was little difference in the resulting bumblebee contact network structure. If multiple bees were recorded within the minimum distance at the same time, all possible pairs of interaction partners were recorded. Multiple interactions were recorded between pairs if they moved out of, and back in to, the minimum distance. This proximity interaction approximates recording direct physical contact between pairs of bees and was therefore informative of interactions that could transmit information or disease.

2.5.3.2 Head-to-head Interactions

The second detection technique aimed to detect antennation interactions between pairs of bees. Antennation in bumblebee colonies is known to contribute to the social organisation of the colony by communicating information related to the dominance status of the individuals involved (Hogeweg and Hesper, 1983; van Honk et al., 1981). This detection technique does not directly detect antennal contact; therefore it will be more accurately termed a 'head-to-head' (HTH) proximity interaction. In this technique, the antennae of each bee were modelled *in silico*, and an algorithm was developed to detect when an antennation interaction could have occurred according to the arrangement of the model antennae. First, the range of movement of each bee's antennal tips was modelled as a polygon approximating an annular sector, which was referred to as the 'interaction zone' (Figure 2-19). The centre of the annulus, from which the sector was derived, was the centroid of each detected tag. For each bee, the radius of the larger circle R of each annulus was 1.6 times the width of that bee's thorax. This scaling factor was determined by measuring the distance between the tag centroid and the antennal tips relative to the thorax width of a sample of bees. The radius of the smaller circle r of each annulus was 90% of the radius R. The sector of each annulus, with inner radius r and outer radius R, was then defined by the central angle ϑ; the angle of ϑ used was 90°. Images of a sample of marked bees were used to determine the appropriate inner angle ϑ that would produce an annular sector to cover the range of antennal movement. Another simple custom MATLAB app was used to display the centroid of the tag (C) in each image by tracking, while the user marked the position of the tip of each antenna (A_1 & A_2) using the cursor. The angle $\angle A_1CA_2$ measured from the sample images suggested 90° was an appropriate inner angle to define the

sector of each annulus (see Figure 2-19). The centre of the outer (and inner) arc of each annular sector was centred on the anterior-posterior axis of each bee and oriented to the anterior end. The result was an interaction zone in front of the head of each bee that described the spatial occupancy of the antennae. Interaction zones were modelled dynamically for each bee in each video frame using custom MATLAB scripts. The scripts modelled each annular section as a polygon with 10 sides for each arc to approximate a curve.

The algorithm for detecting HTH interactions was based on two conditions of the spatial relationships between interaction zones. The first condition was overlap in the polygons that defined the interaction zones of any pair of bees. The second condition was that the major angle of the intersection of the anterior-posterior axes (α) of the pair of bees was greater than 120° (Figure 2-19). For any pair of bees with overlapping interaction zones, the angle α ensured that they were facing each other before the overlap was recorded as a HTH interaction. These conditions were an attempt to describe antennation interactions based on individual interaction zones (see Figure 2-20). While these conditions are able to detect true antennation interactions in some cases, there were many more false positives than true detections (S. Duckerin, personal observation). False positives were unavoidable in this interaction zone based system because of the complex three-dimensional nature of the bumblebee brood pile. For example, bees often incubate brood while their head and antennae are dipped down into crevices within the brood pile (e.g. bee #257 in Figure 2-20), but the interaction zone remains upright and active and can count instances of head-to-head proximity with other nearby interaction zones. In the conditions of

the bumblebee colony, it was not possible to distinguish true antennation interactions from simple cases of head-to-head proximity using this method.

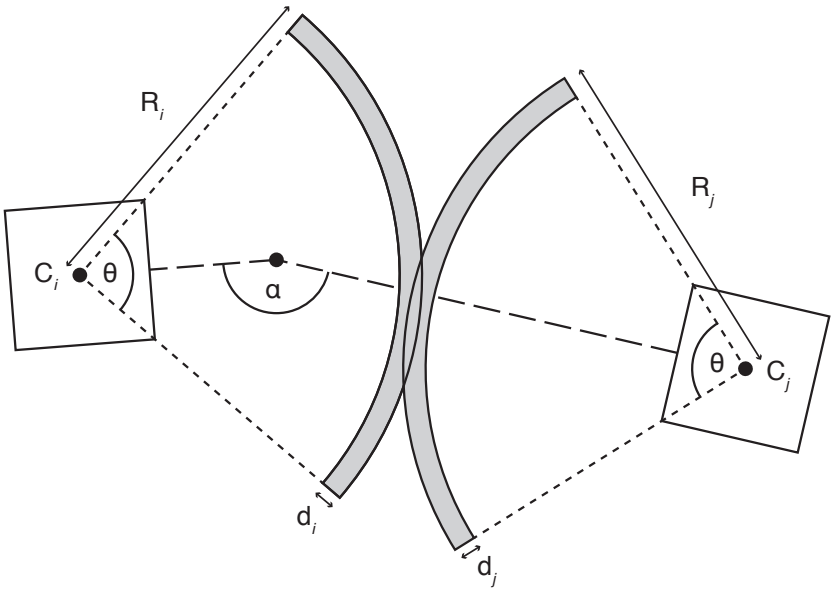

Figure 2-19. Head-to-head interaction zone and interaction detection rules. Illustration of the 'interaction zone' and the rules used to detect antennation interactions between pairs of bees. The interaction zone is defined as an annular sector. Squares represent the tags of two bees i and j. The tag centroid C is shown by a black dot. The 'interaction zone' of each bee is shown as a filled grey annular sector. The annular sector is defined by the outer radius R, the distance d (10% of R), and the inner angle ϑ. The geometric interaction rules required the intersection between two interaction zones, plus the angle $\alpha > 120°$. The angle α was defined as the major angle of the intersection between the vectors created by the anterior-posterior axis of each bee (long-dashed line).

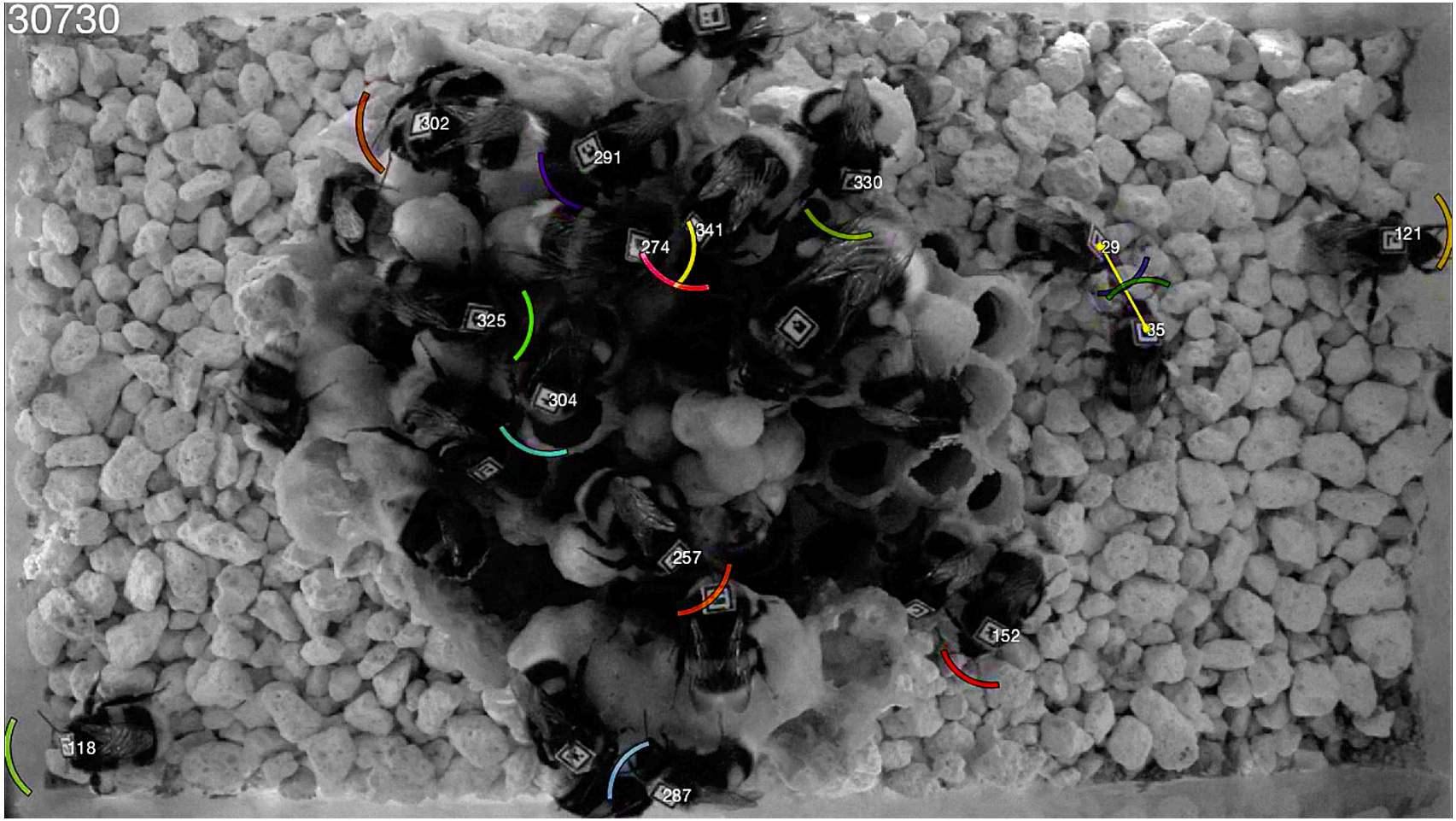

Figure 2-20. Still taken from one of the intranidal videos overlaid with tracking data and individual 'interaction zones'. Numbers next to tags show individual bee IDs. Coloured annular sectors show the interaction zone of each bee. The yellow line connecting bee #29 and #85 represents an automatically detected head-to-head interaction. The number in the top left corner is the frame number (out of 90,000 = 1 hour at 25 fps).

2.6 Discussion

Automated visual tracking systems such as BEEtag are clearly able to generate vast quantities trajectory data at a high spatiotemporal resolution. However, visual tracking in a complex environment is not perfect and raw data must be pre-processed with care to ensure that the results produce precise and accurate interpretations of behaviour.

The 16-bit version of BEEtag performed well, but there are some limitations to working with this system. The error processing described here revealed the presence of a number of false tag detection errors that would have caused significant errors in measurements of behaviour. This issue seems to have been attributable to a relatively small number of tags (see Figure 2-6); therefore, any future users of the16-bit system should avoid these error-prone tags. Following all of the error processing steps above does not guarantee that all false tag detection errors were removed, but any remaining errors are probably not significant as the behavioural results in Chapter 3 and Chapter 4 did not show any obvious unexplained outliers.

One significant limitations of the BEEtag system is the processing time required for complex, high-resolution images (this is also true of the 25-bit system, see Crall et al., 2015). The BEEtag tracking of a 1-hour video from this study completed in approximately 5 days on a laptop computer (MacBook Pro™, 2.8 GHz processor, 16 GB RAM). For this reason, the remainder of the tracking was completed remotely on the University of Bristol high-performance computing (HPC) cluster (BlueCrystal Phase III). Without access to HPC resources such as this, the computational time for tracking high-resolution video would be a serious limiting factor in the practical use of this system. Another factor affecting processing time is

sampling effort. The total observation time and the image recording frame rate will both affect the volume of image data generated by an experiment, and thus the processing time. Future studies should consider the spatial and temporal resolution required to address the biological question being asked.

The open-source availability of the BEEtag software makes it freely available for any user to download and edit. This important feature was a significant factor in deciding to adopt the BEEtag system over other, commercially available, tracking systems. Any user can suggest updates via the repository on the GitHub website (https://github.com/jamescrall/BEEtag). A future aim of this work will be to submit the 16-bit customisation and some of the pre-processing functions to the BEEtag GitHub repository for others to use.

The interpretation of behaviour in this context relied on user designed algorithms. As an alternative, the use of machine learning (ML) algorithms in the interpretation of behaviour from complex data is an exciting future direction in behavioural ecology. The ML approach begins with a trained observer manually labelling behaviour in a small training dataset. The labelled data is used to 'train' an automated behaviour classifier to detect the labelled behaviour based on complex multivariate data. The goal is to automatically generate a behaviour detection algorithm that is more accurate than any algorithm that could be written by a human. This potential of this approach in advancing social insect research was demonstrated in a recent paper detecting social interactions in honeybees (Blut et al., 2017). These authors used a visual tracking system (Mersch et al., 2013) to generate trajectory data of groups of honeybees, and trained a behaviour classifier to identify 'encounter' behaviours between bees (antennation, offering, begging and trophallaxis) directly from the trajectory data. The classifier correctly

detected 93% of encounters in the testing phase, but 13% of detected encounters were false positives. Such an approach could be applied to detect more subtle interactions within bumblebee colonies, which would be an exciting area of future research.

The result of this chapter was over 200 million video tracking data points that describe high-resolution bumblebee movement trajectories. This incredibly rich dataset was used primarily to track the locomotor behaviour and foraging behaviour of individual bee (Chapter 3) and to detect social interactions between bees (Chapter 4). There are undoubtedly many more intricate and interesting biological patterns that can be described by this technique and I expect to see much more automated behavioural data collection in the future.

Chapter 3

Socially Mediated Pesticide Exposure Risk and Impacts on Task Allocation

3.1 Introduction

Division of labour is a defining characteristic of social insects that allows colonies to efficiently perform tasks in parallel through individual task specialisation (Oster and Wilson, 1978). When individuals are able to switch tasks in response to local changes in demand, the global allocation of workers across various tasks (task allocation) becomes flexible and colony patterns of division of labour can shift over time (Gordon, 1996). This flexibility confers the system with great robustness in the face of environmental fluctuations (Gordon, 2002b), demographic disruptions (Bloch and Robinson, 2001; Crall et al., 2018) and shifting colony needs (Robinson et al., 2009b; Seeley, 1989). Colony responses to natural disruptions have been well studied, but social insects are also faced with many emerging anthropogenic threats including climate change, habitat loss and exposure to agricultural pesticides (Vanbergen and Insect Pollinators Initiative, 2013). It is not fully understood how social insect colonies are affected by such threats today, nor how they will respond in the future (Chapman and Bourke, 2001; Sponsler and

Johnson, 2017). Many social insects provide vital ecosystem services such as seed dispersal, pest control, and pollination (Del Toro et al., 2012; Klein et al., 2007; Noriega et al., 2017; Sumner et al., 2018). If we are to conserve these species and the services they provide, it is crucial to understand how complex social insect colonies respond to these emerging anthropogenic threats.

The flexibility of task allocation in social insect colonies is possible because the process is decentralised and self-organising (Gordon, 1996). There is no centralised control of the organisation of work; independent workers modulate their behaviour according to simple behavioural rules in response to local social and environmental information (Camazine et al., 2001). The collective behavioural responses of individual workers to local information defines colony-level behaviour (Pinter-Wollman, 2012), which in turn alters the state of the colony and the information available to individuals, thus creating a system of feedback between the behaviour of the individual and the collective behaviour of the colony (Gordon, 2016). Distributed processing allows the colony to respond flexibly to both shifting demands and significant disruptions by reallocating workers where and when effort is needed (Charbonneau and Dornhaus, 2015; Crall et al., 2018; Seeley, 1989). Such processes of feedback and interdependence create systems that display nonlinearity in the emergence of complex global attributes (Bar-Yam, 1997b), i.e. the collective behaviour of the system is more complex than the sum of the behaviour of the system's parts. The corollary of nonlinearity in social insect systems is that it is not possible to fully understand colony organisation by merely studying the isolated behaviour of individual insects. Therefore, studying the colony as a complex system can further the

understanding of the mechanisms underlying colony social organisation and may help in predicting the responses of colonies to changing environments.

Experimental disruptions are often used to test the limits of the remarkable ability of social insect colonies to produce both flexible and robust systems of social organisation. Some colony responses to disruption include efficient emigration and reproducible patterns of task allocation following nest destruction (Sendova-Franks and Franks, 1994), fast food distribution following starvation (Sendova-Franks et al., 2010), reallocation of workers to replace the loss of foragers or nest-workers (Bloch and Robinson, 2001; Crall et al., 2018; Robinson et al., 2009b). These experiments aim to simulate natural disruptions, the results of which strengthen the view that these strategies are adaptive and allow social insect colonies to react to the challenges of their environment.

Social insect colonies that live in modern agricultural landscapes face a range of novel anthropogenic challenges such as destruction of habitat, introduction of novel pests and pathogens and exposure to pesticides; all of which are considered to be pressures acting on social insects from the level of the individual to the level of the population (Alkassab and Kirchner, 2017; Vanbergen and Insect Pollinators Initiative, 2013). Non-target exposure to systemic neonicotinoid insecticides poses a particular risk to social pollinators because these chemicals are widely used around the world and are present in the pollen and nectar of treated crops (Heimbach et al., 2017). Neonicotinoid exposure has been shown to have a range serious negative effects on the behaviour of individual social bees and colony productivity (see Godfray et al., 2014; Godfray et al., 2015), but we do not fully understand how colonies fail and what factors affect colony sensitivity or robustness. It is often assumed that all individuals within colonies are evenly exposed, thus

experience equal toxic effect (Alkassab and Kirchner, 2017), but the behavioural responses of individuals from across functioning colonies have not been tested.

The movement of individual insects within the colony and in the external environment, i.e. their locomotor behaviour, underpins many aspects of individual and social behaviour in *Apis* and non-*Apis* bees. Self-organisation within the colony relies on active, interacting individuals able to sample local information and respond appropriately (Beshers and Fewell, 2001). Individual locomotor behaviour relies on communication between the nervous system and the muscular system. Neonicotinoids directly affect locomotor behaviour by acting as acetylcholine receptor agonists in the insect central nervous system, causing hyperactivity, trembling, uncoordinated movement, and ultimately death (Moffat et al., 2016). It is possible that sub-lethal effects on the nervous system could disrupt colony functioning by impairing individual locomotor behaviour, but we do not yet understand how these effects on individuals scale up to effects on the colony. Without a mechanistic understanding of colony failure our ability to assess exposure risk in the field and to make predictions is limited (Sponsler and Johnson, 2017).

3.1.1 Effects of Neonicotinoids on Locomotor Behaviour in Bees

Individually caged honeybees (*Apis mellifera*) exposed to a single dose of neonicotinoid (0.1-2 ng/bee) show acute increases in locomotor activity (recorded as distance moved and time spent immobile; clothianidin, Alkassab and Kirchner, 2018; imidacloprid, Lambin et al., 2001). It is thought that the observed stimulation of motor activity at low doses and during acute exposure is a result of neonicotinoid-induced neuronal activation (Teeters et al., 2012). The same doses (1-2 ng/bee) can also have negative effects on

postural control (time spent upside down and frequency of upside down; Alkassab and Kirchner, 2018). Chronic low doses can also induce impaired postural control. Williamson et al. (2014) exposed groups of 10 honeybee workers to independent treatments of four different neonicotinoid pesticides (dinotefuran, thiamethoxam, imidacloprid, clothianidin) over a period of 24 h before behavioural testing. The concentration used in this study was 2-3 ppb (ppb = parts per billion, equivalent to μgL^{-1}), which translated to approximately 0.32-0.48 ng/bee/24h, which also showed negative effects on the same metrics of postural control as above, but no effect on walking, inactivity or flying. Higher acute doses have been found to reduce the total walking distance covered by individually caged honeybees (topical dose 3.8 ng/bee thiamethoxam, Charreton et al., 2015; 2.5-20 ng/bee imidacloprid, Lambin et al., 2001). Additionally, exposure to nectar and agar containing imidacloprid at concentrations of 50 μgkg^{-1} resulted in reduced distance moved by individually caged honeybees over 24 hours (Teeters et al., 2012). This time/dose-dependent effect of neonicotinoids on locomotor behaviour demonstrates that initial hyperactivity can lead to a more general toxic effect on behaviour over longer time scales and higher doses.

Bumblebee (*Bombus terrestris*) locomotor activity has also been shown to have a dose-dependent relationship with neonicotinoid exposure. Cresswell et al. (2013) found that exposure to a nectar food source containing imidacloprid at a concentration of 98 ppb (much higher than typical field realistic concentrations) over 8 days reduced the distance moved by individually caged bumblebees. In another paper, Cresswell et al. (2012) however found no effect on the distance moved per hour of individually caged bumblebees after 4 days of exposure to dietary imidacloprid at a wide range of concentrations (0.0-125 ppb). This is surprising given that imidacloprid and thiamethoxam at 100

ppb have each been found to cause severe mortality in bumblebee microcolonies (Mommaerts et al., 2010; Tasei et al., 2000). This disparity in the effects on bumblebees could be attributed to either some influence of the social context of microcolonies or the metabolic cost of flight (3m foraging distance in Mommaerts et al., 2010). Neither of these possibilities have explored in the literature. Finally, Cresswell et al. (2012) also monitored locomotion in groups of 10 *A. mellifera* workers during the same experiment and found no effect. These mixed results between species and social contexts suggest current tests may not be able to describe the full range of locomotor deficits bees may face when they are at work within a queenright colony setting. Reduced locomotor activity could limit the range of movement of bees inside the nest, which could act to reduce the rate at which individual bees are able to sample location specific sources of information (e.g. brood signals, nectar pots). The severity of the consequences of decreases in the ability to move through space will be expected to be different according to an individual's task. For tasks such as incubating or feeding brood, workers do not need to cover significant distances to remain engaged. On the other hand, the consequences for the regulation of foraging activity, which requires high levels of activity inside the nest (Dornhaus and Chittka, 2001), could be much greater.

These effects on locomotor activity and coordination have been linked to reported effects of pesticide exposure on foraging activity, efficiency and success (Alkassab and Kirchner, 2018). Individual foragers can suffer directly from neonicotinoid-induced homing failure. An acute field realistic dose of thiamethoxam caused individual *A. mellifera* foragers to suffer increased mortality by failing to return to the colony (Henry et al., 2012). Neonicotinoid exposure can also reduce the efficiency of successful foraging

bouts. Acute exposure of honeybee foragers to imidacloprid (≥1.5 ng/bee) and clothianidin (≥0.5 ng/bee) reduced their foraging activity and increased foraging bout duration (Schneider et al., 2012). Chronic exposure of queenright *B. terrestris* colonies to dietary thiamethoxam (2.4 μgkg^{-1}) also caused a significant reduction in the colony-wide number of foraging bouts plus an increase in foraging bout duration (Stanley et al., 2016). Similar impairments on pollen foraging have been described during chronic imidacloprid exposure in bumblebees. *B. terrestris* colonies exposed to chronic dietary imidacloprid (10 μgkg^{-1}) suffered increased foraging bout duration, smaller pollen load sizes and an increase in unsuccessful pollen foraging bouts (Gill and Raine, 2014; Gill et al., 2012). Pollen foraging was also impaired in bumblebees measured after a 14-day exposure to imidacloprid in pollen (0.7 μgkg^{-1}) and nectar (6 μgkg^{-1}), but there was no significant effect on nectar foraging rate (Feltham et al., 2014). No effect on the foraging ability of *Bombus impatiens* foragers was identified during chronic exposure to dietary imidacloprid at 7 μgkg^{-1}. However imidacloprid at 30 μgkg^{-1} exposure increased flower-handling time (in a study funded by Syngenta and Monsanto; Morandin and Winston, 2003). Neonicotinoid exposure can also affect the social regulation of foraging behaviour. Gill et al. (2012) found that the number of foragers in exposed bumblebee colonies actually increased, suggesting a colony response to individual forager impairment. In conclusion, neonicotinoid exposure can affect bee foraging behaviour by reducing the rate of successful foraging bouts, but colonies may retain the flexibility and robustness for a degree of compensation.

Another important component of understanding the risks of neonicotinoid exposure is the potential for individuals and colonies to recover from a dose of pesticides. Recovery for individuals and colonies could be environmentally

relevant in agricultural systems that include large areas of mass flowering crops such as oil seed rape. When such bee-attractive crops bloom in synchrony across the landscape they provide an abundant food source for bees. If these crops have been treated with a systemic neonicotinoid insecticide, foraging bees could face a short pulse of neonicotinoid exposure during the flowering period (2-3 weeks). Free-foraging bees will return to foraging on mixed floral resources when the mass flowering period is over (Heinrich, 1979). Individually-caged bumblebees exposed to a 3-day pulse of imidacloprid recovered locomotor activity after 24 h (Cresswell et al., 2013). Early-stage bumblebee colonies showed a dose-dependent recovery in brood production following a 14-day pulsed exposure to imidacloprid (Laycock and Cresswell, 2013). These results suggest that individual and colony-level behaviour may be able to recover from pulsed exposure regimes. However, in larger colonies, nectar pots could act as significant reserved of pesticides inside the colony. Ramirez-Romero et al. (2005) found honeybees showed only a partial recovery in foraging behaviour following a pulse of nectar containing 48 ppb imidacloprid. These studies have not considered the comparative recovery of nest workers and foragers. Honeybee foragers have reduced immunity compared to nurse bees (Amdam et al., 2005), but they show high expression of immunity and detoxifications genes during nectar processing (Vannette et al., 2015). This raises the possibility that task groups of bees could respond differently to detoxification.

3.1.2 Inter-individual Behavioural Variation and Task Allocation in Bumblebees

The activity levels of individual bumblebees inside the nest are influenced by patterns of task allocation. For example, successful returning foragers will run

"excitedly" throughout the inside of the nest, stopping briefly to unload their forage into a nectar pot before leaving the nest to forage once again (Dornhaus and Chittka, 2001). This high-activity intranidal (within the nest) behaviour exhibited by foragers is thought to help distribute a pheromone and to increase social contacts with other bees, which can stimulate other workers to begin foraging (Dornhaus and Chittka, 1999; Dornhaus and Chittka, 2004). Individuals characterised by locomotor inactivity (i.e. 'lazy' workers not engaged in any task) are also common in bumblebee colonies and show consistency in inactivity over days (Jandt et al., 2009). The exact role of inactivity in bumblebees is unknown (Jandt et al., 2012), but they may act as thermoregulatory reserves (Weidenmüller, 2004) or they may selfishly rest and build fat reserves to increase their chances of egg-laying following the senescence of the queen (Jandt and Dornhaus, 2011). Variation in the circadian rhythm of locomotor activity is also linked to task allocation in bumblebees. Foragers exhibit regular diurnal circadian rhythms, whereas nest bees do not and will remain active throughout the night (Yerushalmi et al., 2006). These alternative circadian rhythms are endogenous and are determined by size; large callow (newly emerged) workers reared in isolation show rhythmicity, whereas small callow workers do not (Yerushalmi et al., 2006). The possibility of pesticide-induced disruptions to individual locomotor activity could therefore have significant consequences on some of the processes that regulate task allocation.

The spatial organisation of bumblebees inside the nest is also linked to task allocation. The construction of the brood pile in bumblebee nests is unpredictable (Cameron, 1989), but individual workers arrange themselves in a non-random way inside the nest (Crall et al., 2015; Crall et al., 2018; Jandt and Dornhaus, 2009). The queen consistently occupies a relatively small area

of the nest near the centre of the brood, while workers exhibit high variation in space-use patterns (Jandt and Dornhaus, 2009). Smaller workers also tend to be central and help care for the brood, whereas larger workers tend to patrol the periphery and will also leave the nest to forage (Jandt and Dornhaus, 2009). Although workers show only weak specialisation to tasks, they do maintain spatial fidelity zones within the nest throughout their lives (Jandt and Dornhaus, 2009). This spatial sorting affects the local information that individual bees are exposed to, which in turn influences their behaviour and will feedback into colony-level task allocation (Crall et al., 2018).

Foraging behaviour is a complex behavioural task associated with high energetic demands on the individual. Successful bumblebee foragers must navigate over large spatial scales in a complex landscape, remember disparate and ephemeral food sources and handle a range of flower morphologies (Lihoreau et al., 2012; Woodgate et al., 2016; Woodgate et al., 2017). Individual cognitive or motor impairments could therefore have significant effects on the foraging success of individual bees. Foraging however is also socially regulated. When colony-level foraging is impaired, non-foraging individuals inside the nest can respond to foraging-related information and switch to becoming foragers themselves (Crall et al., 2018). If pesticide exposure reduces the ability of bees to respond to such information, then the flexibility of task allocation could be affected. To date, studies regarding the effects of pesticides on social bee foraging have only measured the activity of foragers outside the nest and considered the impact of reduced food intake on colony development (Feltham et al., 2014; Gill and Raine, 2014; Gill et al., 2012; Morandin and Winston, 2003). Of these previous studies, there have been mixed behavioural results that are difficult to interpret when compared to studies of the effects on individual bees (e.g. Stanley and Raine, 2016). A

missing component of our understanding of the risks of non-target pesticide exposure is the behavioural response to exposure of all the individuals that make up the colony (Heimbach et al., 2017). Worker behaviour and colony behaviour are interlinked, thus an integrated approach to understanding pesticide exposure risk is needed.

3.1.3 Aims & Hypotheses

The primary aim of this study was to measure the effects of field-realistic neonicotinoid exposure on the behaviour of bumblebee workers integrated within the social setting of queenright colonies. Further to this, the secondary aim was to record the effects of exposure on behaviour with respect to functional task groups (active foragers and non-foragers). Finally, this study also aimed to track changes in the allocation of workers across functional task groups during exposure. This approach leads to the following specific hypotheses.

3.1.3.1 Hypothesis 1: Foraging activity will decrease during pesticide exposure

The first hypothesis states that pesticide exposure will decrease the foraging activity of bumblebee colonies during pesticide exposure. Bumblebee foraging activity has been shown to be negatively affected in previous studies (e.g. Feltham et al., 2014; Gill and Raine, 2014; Gill et al., 2012), but it is still relevant here because foraging will be used to define task groups to address subsequent hypotheses. This hypothesis was broken down into multiple parts. Foraging is a complex and demanding task; therefore the first foraging hypothesis states that pesticide exposure will reduce the rate of foraging, i.e. total number of foraging bouts per colony (Hypothesis 1a). The rate could be

affected by changes to the number of foragers engaged in foraging; therefore the second foraging hypothesis was that the number of foragers would decrease during exposure (Hypothesis 1b). Alternatively, if the number of bouts was stable and the number of foragers increased this would provide evidence of a colony level response to low food intake, as described by Gill et al. (2012). The final two forager-related hypotheses focus on individual level foraging activity. The first individual level hypothesis states that that foraging bout duration will increase during exposure (Hypothesis 1c). The second advances on previous studies to consider the behaviour of foragers inside the nest and states that the time spent inside the nest in between foraging bouts (the inter-bout duration) will also increase during exposure (Hypothesis 1d). Finally, each of these hypotheses was followed up with the additional hypothesis that any effect of pesticide exposure would recover post-exposure.

3.1.3.2 Hypothesis 2: Task groups will show differences in locomotor behaviour

Many previous studies have described the behaviours of task groups in bumblebees (e.g. Dornhaus and Chittka, 2001; Jandt and Dornhaus, 2009; Jandt and Dornhaus, 2011; Jandt et al., 2009; van Doorn and Heringa, 1986; Yerushalmi et al., 2006), but it was important to test that the specific categorisation of task groups used in this chapter reflected distinctions that exist in real colonies and that the automatically recorded locomotor behaviours were able to describe these distinctions. The first hypothesis stated that active foragers would have higher movement speeds than non-foragers (Hypothesis 2a). Next, space use was also considered as a component of locomotor behaviour that is known to vary in bumblebee task groups,

leading to two specific hypotheses: active foragers will occupy more peripheral nest regions than non-foragers (Hypothesis 2b) and active foragers will occupy larger areas of the nest than non-foragers (Hypothesis 2c).

3.1.3.3 Hypothesis 3: The effects of pesticides on locomotor behaviour will vary according to task group

The final, and key hypothesis this study aimed to test was the possibility that by engaging in different tasks, individuals will experience the toxic effects of pesticides in different ways. Movement speed was the first locomotor behaviour to be considered, which was hypothesised to decrease during pesticide exposure for the whole colony on average (Hypothesis 3a), and was also hypothesised to decrease more strongly for active foragers (Hypothesis 3b). Next, in terms of space use, there was no specific directionality concerning the expected effects on central vs. peripheral nest occupancy; therefore, the null hypothesis states that there will be no effect of pesticide exposure on central vs. peripheral nest occupancy on average (Hypothesis 3c). Additionally, there will be no differences in effect of pesticides on central vs. peripheral nest occupancy with respect to task group (Hypothesis 3d). The second component of space-use tested was nest occupancy area (per observation window), which is more directly related to movement speed; therefore, bees on average will occupy smaller nest areas during pesticide exposure (Hypotheses 3e), and the effect of pesticides on nest area occupancy will be more severe for active foragers than non-foragers. Once again, each of these hypotheses was followed up the additional hypothesis that any effect of pesticide exposure would either recover on average, or would recovery equally with respect to task group.

3.2 Methods

3.2.1 Bumblebee Colonies

Ten bumblebee colonies (*Bombus terrestris audax*) were acquired from BioBest N.V., Belgium. Each colony was standardised to contain a queen and 50 workers to control for the effect of colony size on individual and colony-level behaviour. All colonies were maintained in laboratory conditions at 24°C.

All adult bees and the queen were individually marked with a unique barcode-like visual marker generated by a customised version of the automated BEEtag video-tracking system (Crall et al., 2015). Tag design, technology, and implementation are described in Chapter 2. Colonies were inspected daily for any newly emerged bees, which were marked with a new tag and returned to the colony. Any dead workers were removed from the colony. At the end of the experiment, colonies were terminated by placing them in a freezer at -20°C. The thorax width of individual bees was measured post-mortem to the nearest 0.01 mm with digital callipers as a record of body size (Jandt et al., 2009).

Each colony was housed in a transparent acrylic nest box (180×100×100 mm) connected via a short plastic tube (15 mm diameter, 20 mm length) to a smaller acrylic vestibule (80x100x100mm), which served as a waste deposit area for the colony (Pomeroy and Plowright, 1980). The nest box and the vestibule were both ventilated by small holes around the top edges of the acrylic walls and were lined with cat litter granules to absorb moisture. Furthermore, both boxes were housed within a larger cardboard blackout box (see Figure 2-2) to mimic the darkness of a natural subterranean bumblebee nest (Benton, 2006). The vestibule was connected via a plastic tube (15 mm

diameter, 300 mm length) to a foraging arena (700x500x300mm). Bees were able to leave the nest via the tube and fly freely inside the arena at any time. Nectar (sugar water 50% vol/vol) was supplied *ad libitum* at a gravity feeder inside the foraging arena. The lid of the arena was a transparent acrylic sheet to permit observation and provide lighting for foragers. Lighting was provided on a 12-hour light/dark regime by UV-enriched full-spectrum bulbs to simulate natural daylight (Whitney et al., 2009). Honeybee pollen was supplied *ad libitum* in a small dish inside the vestibule.

3.2.2 Experimental Design

Half of the colonies were assigned to the pesticide treatment group (Colony F, I, K, M & O) and were to exposed to the neonicotinoid pesticide imidacloprid, while the other half were not exposed to any pesticides, thus acting as a control group (Colony G, H, J, L & N). The experiment was conducted on two colonies at a time (one control and one treatment) and was replicated on five separate pairs of control/treatment colonies between November 2016 and August 2017. The experimental schedule ran for 19 days for each control and treatment pair.

Pesticide treatment was delivered according to a baseline-experiment-reversal design (Figure 3-1). The first five days of the experiment were the 'baseline' and all colonies were fed untreated nectar. The following seven days were the 'experiment' and treatment colonies were exposed to imidacloprid-treated nectar (details below). The final seven days were the 'reversal' and treatment colonies were returned to feeding on untreated nectar. Control colonies were fed untreated nectar throughout the experiment.

Behavioural sampling of control and treatment colonies was conducted in three 5-day phases that overlapped with the baseline-experiment-reversal

design: Phase 1 sampling was on all five days of the pre-exposure baseline (day 1-5); Phase 2 sampling was on the last five days of the pesticide exposure experiment (day 8-12); Phase 3 sampling was on the last five days of the post-exposure reversal (day 15-19) (see Figure 3-1). Behavioural sampling was conducted for 1 hour each day, resulting in 15 hours of sampling over three 5-day phases, across 19 days total (Figure 3-1). The two-day gap between behavioural monitoring phases was introduced to allow the pesticide time to take effect, which would help to delineate the effects of each part of the baseline-experiment-reversal design.

The neonicotinoid pesticide imidacloprid was used for the exposure experiment because it is one of the most widely used pesticides in the world (Jeschke et al., 2011) and has previously been shown to be toxic to bees (see Alkassab and Kirchner, 2016a). Imidacloprid was sourced from Sigma-Aldrich at ≥98.0% purity. An initial stock solution was made by dissolving the imidacloprid powder in water at a concentration of 200 μg/L. Treated nectar was prepared by adding an aliquot of 10 μl stock solution to 200 ml nectar to produce a final concentration of imidacloprid at 10 $\mu g/L^{-1}$ (10 ppb). This concentration has been used by several experimental studies and is at the upper end of what is considered to be "field realistic" for imidacloprid in the nectar of treated crops (Bryden et al., 2013; Elston et al., 2013; Gill and Raine, 2014; Gill et al., 2012; Mommaerts et al., 2010; Tasei et al., 2000). Fresh nectar was prepared every few days. The stock solution and nectar solutions were kept refrigerated at 2-4°C while not in use. Neonicotinoids can degrade under UV light (Kagabu and Medej, 1995); therefore, to minimize this effect all gravity feeder tanks were coated in matt black tape. The feeders in control colonies were also covered to maintain a visually identical foraging environment. *B. terrestris* foragers display innate floral colour preferences

(Lunau et al., 1996), which could have affected foraging behaviour in relation to feeders with and without black tape.

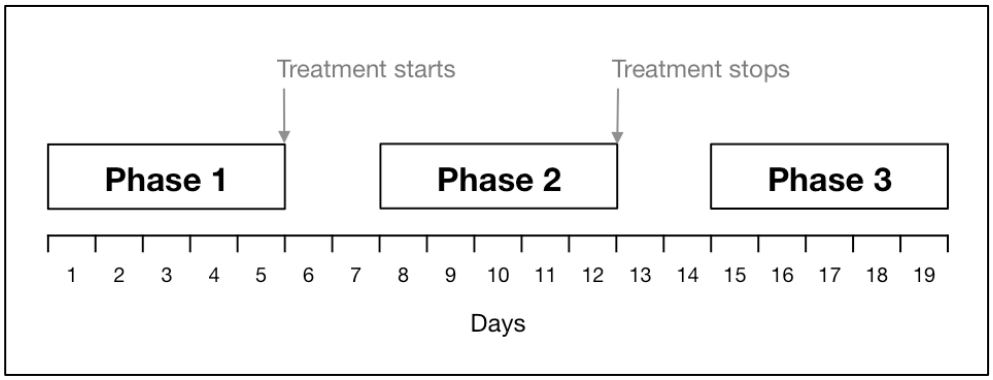

Figure 3-1. Baseline-experiment-reversal experimental design. Pesticide exposure schedule for colonies in the treatment group. Behavioural monitoring was conducted in three 5-day blocks: Phase 1 = baseline monitoring phase, Phase 2 = experimental pesticide exposure phase, Phase 3 = pesticide exposure reversal phase.

3.2.3 Data Collection

Each pair of colonies was monitored in parallel for 1 hour a day. During the 1-hour sampling period, image data was collected in the form of video recordings of behaviour inside the nest (intranidal behaviour) and photos of forager activity at the nectar feeder in the foraging arena. Technical details of image data acquisition are described in Chapter 2.

The tags of individual bees were automatically tracked from the image data using customised functions from the BEEtag package (Crall et al., 2015). The resulting tracking data of each bee consists of a sequence of x-y coordinates that represent their movements through space (a trajectory). Custom MATLAB scripts were used to improve the quality of trajectories and to remove errors in the tracking data (see Chapter 2). Tracking data was used to automatically measure the locomotor behaviour of all individuals inside the nest and the movement of individuals between the nest and the feeder (for full details, see Chapter 2).

The particular aspects of intranidal locomotor behaviour measured were movement speed, spatial centrality and home range. Movement speed was recorded as the median distance travelled per video frame (from the nest video), converted to mm/s. Spatial centrality was measured per day for each bee as the median distance from the social centre of the colony, which was defined as the mean x-y position of the daily intranidal tracking data of all bees from that colony (Sendova-Franks and Franks, 1994). Home range within the nest was recorded by estimating the area of the minimum convex polygon that described 50% of a bee's recorded spatial positions (Crall et al., 2018), know as the "core range" in movement ecology.

Foraging behaviour was quantified automatically from the tracking data of individual bees as their detected tag moved between the nest image data

and the feeder image data (for details, see Chapter 2). This automated technique defined a foraging bout as a round trip that included leaving the nest, visiting the feeder and returning to the nest. The total number of foraging bouts and the number of unique foragers were recorded for each colony. At the individual level, foraging bout duration and the time spent inside the nest between bouts (the inter-bout duration) were also recorded for each bout.

Bees were classified into one of three broad task groups based on the classification system used by Yerushalmi et al. (2006). Within each 5-day phase, bees were classified as either a 'non-forager', an 'intermediate forager', or an 'active forager' based on the sum of their foraging bouts completed during that phase. Non-foragers did not complete any foraging bouts, while active foragers completed at least 10 foraging bouts over five days. The rules defining these classifications were arbitrary, but they create two distinct behavioural groups of bees that can be used to examine the comparative effects of pesticide exposure between active foragers and non-foraging nest workers. Intermediate foragers did not fit into either category. All bees were re-classified within each phase. Different thresholds of the number of foraging bouts used to distinguish active foragers and intermediate foragers were also tested. Threshold values ranging from 5-15 foraging bouts per phase did not appear to significantly affect the comparisons of locomotor behaviour between groups shown in Section 3.3.2.

3.2.4 Statistical Analysis

Statistical analyses were conducted in the R programming environment (version 3.3.0; R Core Team, 2016).

Count variables of foraging behaviour (number of foragers and number of foraging bouts) were modelled using generalised linear mixed models (GLMM) with negative binomial error distributions. The R package 'glmmADMB' was used to construct GLMMs for foraging count data (Fournier et al., 2012). These GLMMs were used to test the fixed effects of experimental group (control or treatment) and phase (pre-exposure Phase 1, treatment exposure Phase 2 or post-exposure Phase 3), plus the interaction between group and phase on colony foraging activity (Hypothesis 1). Colony was included as a random effect in these GLMMs to account for the non-independence of repeated measures on the same colonies over time. The significance of fixed effects were tested using Wald's chi-square test (Type II).

The daily number of bouts per forager was calculated by dividing the daily number of foraging bouts by the daily number of foragers for each colony. The number of bouts per forager was modelled as a continuous response variable using a linear mixed model (LMM). The continuous variables foraging bout duration and inter-bout duration were also modelled with LMMs. Foraging bout duration and inter-bout duration were log transformed to improve model fit. Model output was untransformed as appropriate. As above, the fixed effects included the experimental group, phase, plus the interaction between group and phase (Hypothesis 1).

For models of foraging data measured from colonies (number of foraging bouts, number of foragers, bouts per forager), colony ID was included as a random effect to account for the non-independence of repeated measures on the same colonies over time. For models of foraging data measured from individuals (bout duration, inter-bout duration), individual ID (nested within colony ID) was included as a random effect to account for the non-independence of repeated measures on the same individuals over time.

Separate LMMs were also used to model different components of intranidal locomotor behaviour (movement speed, spatial centrality, and home range). Certain variables were transformed to improve model fit: speed was cube root transformed, home range area was square root transformed. Model output was untransformed as appropriate. In Section 3.3.3, LMMs of locomotor behaviour were used to test the fixed effects of task group (non-forager, intermediate forager or active forager), phase, and the interaction between task and phase on data from control colonies (Hypothesis 2). In the following three sections (Section 3.3.4, Section 3.3.5 and Section 3.3.6), LMMs of locomotor behaviour were used to test the fixed effects of experimental group and phase, plus the interaction between group and phase on the full data set (Hypothesis 3). For these models of locomotor behaviour, individual ID was included as a random effect, nested within colony ID, to account for the non-independence of repeated measures of the same individuals over days. All LMMs were constructed using the R package 'lme4' (Bates et al., 2015). The R package 'lmerTest' was used to provide p values for tests of fixed effects in LMMs using Satterthwaite's method (Kuznetsova et al., 2017).

The relationship between body size and task group allocation was also tested with data from control colonies only. Separate linear models were used to test the relationship within each phase because individual bees were reclassified into task groups for each phase. Models concerning body size excluded queens (N = 5) and any gynes (N = 3). Gynes were defined as any non-queen with thorax width >7 mm.

Models were simplified according to backward stepwise elimination to identify significant predictors of the response variable; predictors were eliminated when they did not improve model fit at critical $p<0.05$.

The R function 'multcomp' (Hothorn et al., 2008) was used to conduct multiple pairwise comparisons on model parameter estimates for specific hypothesis testing. Post-hoc tests were always conducted on full models. This technique sets up Tukey post-hoc contrasts for all pairwise comparisons and calculates adjusted p-values for each test.

3.3 Results

A total of 148 hours of video were analysed from across 10 colonies over the three 5-day phases of the experiment: day 1-5 (Phase 1), day 8-12 (Phase 2), day 15-19 (Phase 3). Due to technical errors, two hours of video were lost: one from the control Colony G on day 19, the other from treatment Colony K on day 5. Automated video tracking generated intranidal movement trajectories of 1072 unique bumblebees across all colonies. The feeder cameras recorded a total of 860,242 images. The foraging bout detection algorithm recorded a total of 5046 foraging bouts completed by 402 unique foragers.

3.3.1 Effects of Pesticide Exposure on Foraging Activity

The results in this section on foraging activity refer to "foragers" as any individual that completed at least one bout (active foragers and intermediate foragers). The intranidal behaviour of the three functional task groups will be discussed in the next section.

There were high daily fluctuations in foraging activity in both control and treatment colonies (Figure 3-2). Total foraging effort did not seem to be affected by pesticide exposure, there was no effect of experimental group (control or treatment) on either the number of unique bees that completed foraging bouts (Hypothesis 1a; GLMM χ^2= 0.587, p=0.444) or on the number of foraging bouts (Hypothesis 1b; χ^2= 2.294, p=0.130). However, there was a

significant interaction between experimental group and phase on the average number of bouts per forager (Figure 3-3**;** LMM F=3.091, p=0.049). Pairwise comparisons of the model estimates showed that within the treatment group there was a significant decrease in the number of bouts per forager between the pre-exposure Phase 1 and the exposure Phase 2 (Tukey post-hoc test, p=0.004). Also, in treatment colonies, the number of bouts per forager was not significantly different between Phase 2 and Phase 3 (p=1.000), suggesting average foraging effort may not recover post-exposure. Comparing the number of bouts per forager between the treatment group and the control group showed that that there were no significant differences between control and treatment colonies during the pre-exposure Phase 1 (p=0.970), during Phase 2 (p=0.053), or during Phase 3 (p=0.195). Despite any direct significant difference between experimental groups, the observed trends in the average number of bouts per forager suggest an effect of pesticide exposure during Phase 2.

Foraging bout duration was not affected by pesticide exposure (Hypothesis 1c). There was no significant effect of experimental group on foraging bout duration (Figure 3-4), i.e. the time spent outside of the nest at the feeder (LMM F=0.400, p=0.545). There was a trend for increasing foraging bout duration over time in both groups, however the effect of phase was not significant (LMM F= 3.343, p=0.062). On the other hand, foragers in treatment colonies spent more time inside the nest in between foraging bouts during pesticide exposure than foragers in control colonies (Hypothesis 1d; Figure 3-5). The effect of the interaction between experimental group and phase on inter-bout duration was not significant (LMM F=2.495, p=0.072), but there was a significant overall effect of experimental group (LMM F=6.043, p=0.044). Tukey post-hoc tests showed that the inter-bout duration

of foragers in treatment colonies during Phase 2 was significantly longer than control colonies during Phase 2 (Tukey post-hoc test, p=0.011).

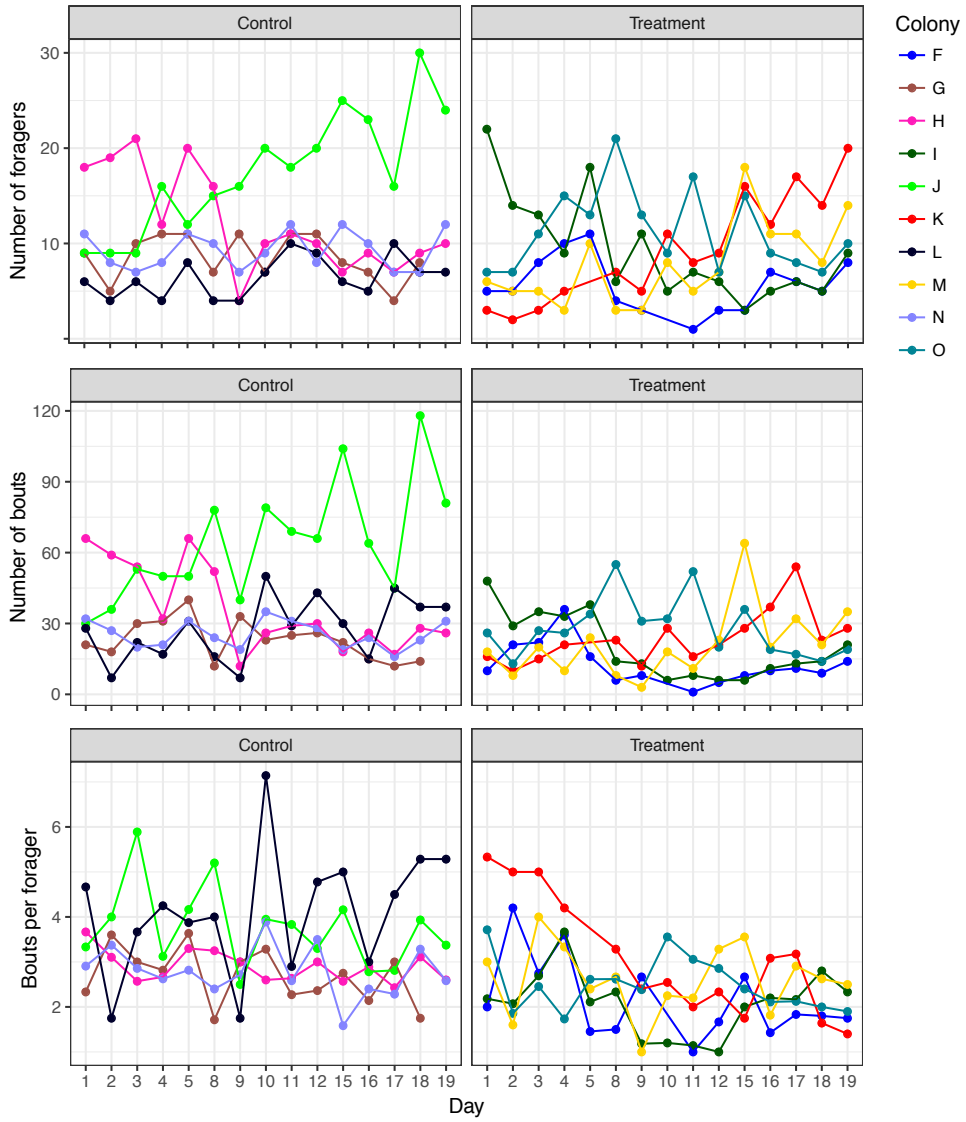

Figure 3-2. Daily variation in foraging activity. Foraging activity is shown as the number of unique foragers (top row), the number of foraging bouts (middle row), and the average bouts per forager (bottom row).

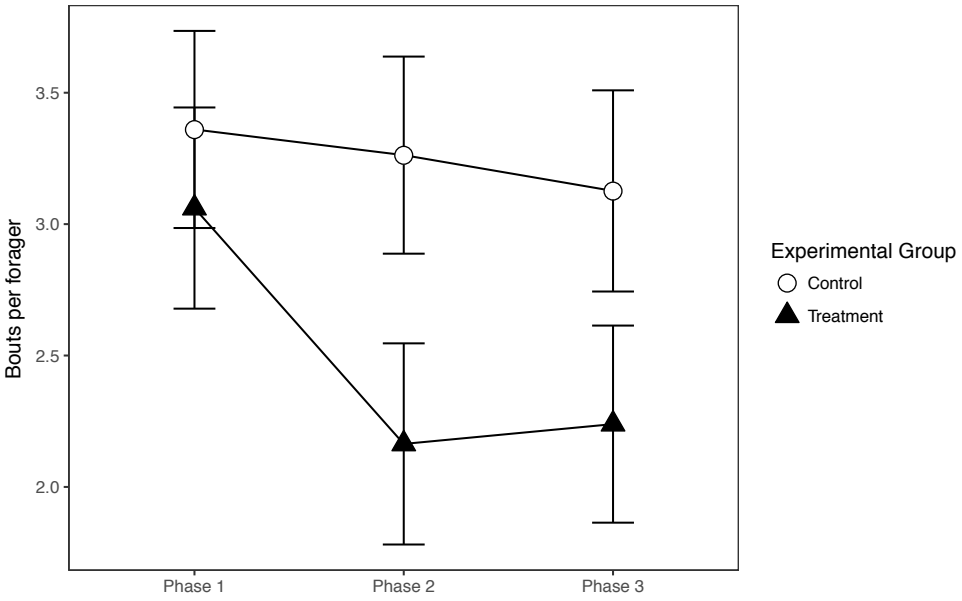

Figure 3-3. Pesticide exposure reduces average foraging effort per bee, which may not fully recover post-exposure. Points show mean number of bouts per forager from control colonies (open circles) and treatment colonies (closed triangles), estimated from a linear mixed model. Error bars represent 95% confidence intervals. There were no statistical differences between experimental groups within phases at $p<0.05$. See Section 3.3.1 for more results.

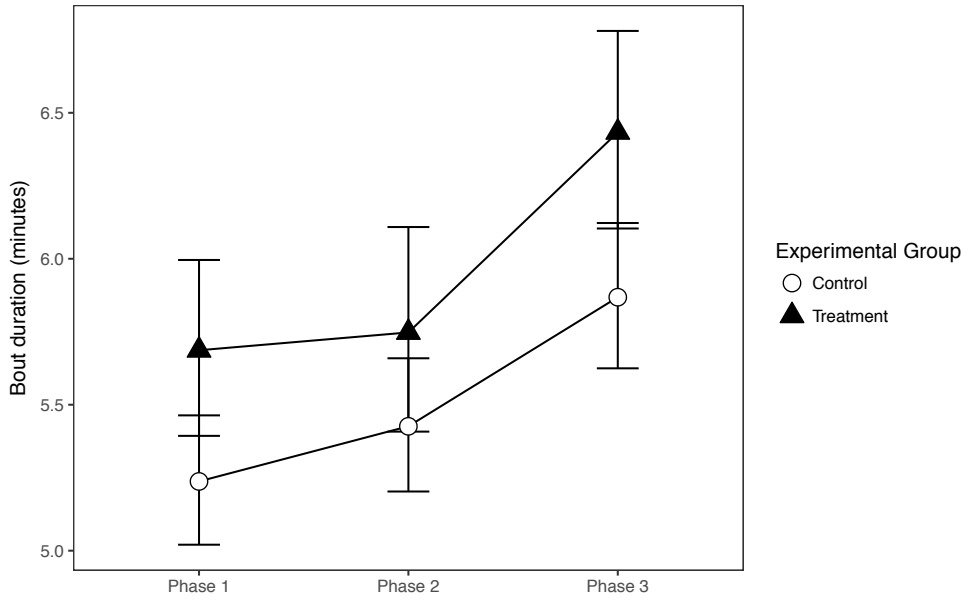

Figure 3-4. No effect of pesticide treatment on the duration of foraging bouts. Points show mean bout duration (time outside the nest while visiting the feeder) of foragers from control colonies (open circles) and treatment colonies (closed triangles), estimated from a linear mixed model. Error bars represent 95% confidence intervals. There were no statistical differences between experimental groups within phases at $p<0.05$. See Section 3.3.1 for more results.

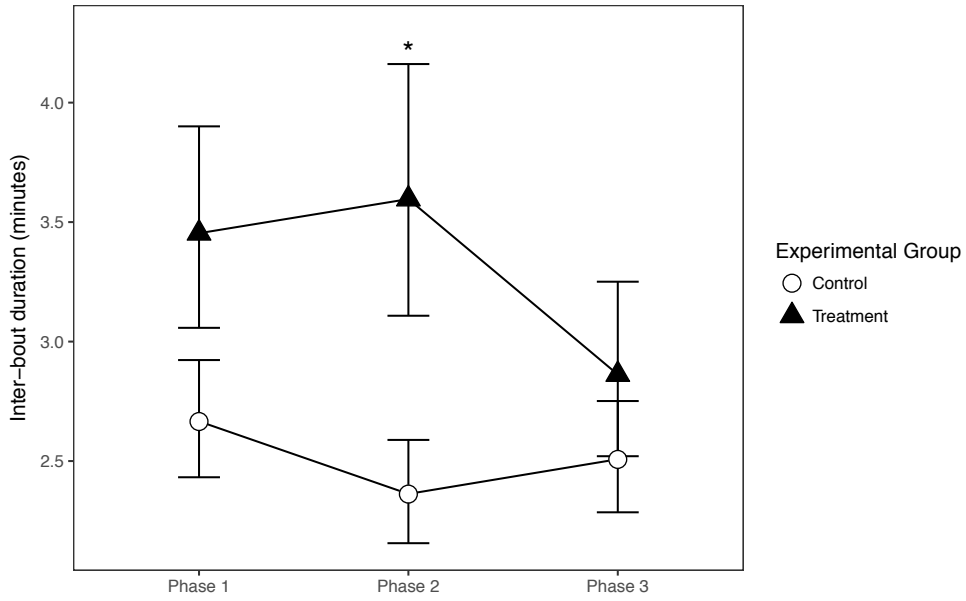

Figure 3-5. Foragers spend longer inside the nest between foraging bouts during pesticide exposure, but they recover after exposure. Points show mean inter-bout duration (time inside the nest between foraging bouts) of foragers from control colonies (open circles) and treatment colonies (closed triangles), estimated from a linear mixed model. Error bars represent 95% confidence intervals. Asterisks at phases denote statistical differences between treatment groups within that phase at $p<0.05$. See Section 3.3.1 for more results.

3.3.2 Task Group Classification

The number of workers assigned to task groups in each phase for control and treatment colonies is shown in (Table 3-1). The proportions of bees in each task group remained stable in control colonies over time. In control colonies, non-foragers made up 68%, 71%, and 74% of the colony in Phase 1, 2 and 3, respectively; intermediate foragers made up 21%, 19% and 19% of the colony in Phase 1, 2 and 3, respectively; active foragers made up 11%, 10% and 9% of the colony in Phase 1, 2 and 3, respectively. In treatment colonies, non-foragers made up 72%, 76%, and 77% of the colony in Phase 1, 2 and 3, respectively; intermediate foragers made up 21%, 21%, and 20% of the colony (Phase 1, 2 and 3); active foragers made up 7%, 3% and 3% of the colony (Phase 1, 2 and 3). A separate chi-squared test of independence was performed to examine the relationship between pesticide treatment and task allocation in each phase. The relation between these variables was significant only during Phase 2 (χ^2=13.941, d.f.=2, $p<0.001$). This result seems to suggest that there were fewer active foragers that completed more than 10 bouts in the treatment group during pesticide treatment, but still a comparable proportion of intermediate foragers engaged in a lower level of foraging activity. Additionally, task group allocation correlated with body size (Phase 1: F=29.606, d.f.=2, $p<0.001$; Phase 2: F=10.258, d.f.=2, $p<0.001$; Phase 3: F=6.143, d.f.=2, p=0.002). In control colonies during Phase 1 and Phase 2, bees classified as non-foragers had significantly smaller thorax widths than both intermediate foragers (Tukey post-hoc test, Phase 1: $p<0.001$, Phase2: $p<0.001$) and active foragers (Phase 1: $p<0.001$; Phase 2: p=0.018). In control colonies during Phase 3, bees classified as non-foragers were only smaller than those classified as intermediate foragers (p=0.002).

In control colonies, bees classified as non-foragers in one phase were the least likely to change to another task group in the next phase (Figure 3-6). Of the non-foragers in Phase 1, 78.1% were still non-foragers in Phase 2 and of those that were non-foragers in Phase 2, 81.5% were still non-foragers in Phase 3. Only half of the active foragers in one phase were still active foragers in the next phase (Figure 3-6). Of the bees classified as active foragers in Phase 1, 50% were still active foragers in Phase 2 and of those that were active foragers in Phase 2, 45.4% were still active foragers in Phase 3. The membership of the intermediate forager group was the least stable. It seems that the task switching patterns of the intermediate forager group changed over time (Figure 3-6).

In treatment colonies, non-foraging workers were also the most stable group (Figure 3-6) with 76.2% and 73.3% remaining in the group between Phase 1 and Phase 2, and between Phase 2 and Phase 3, respectively. The membership of the intermediate forager group was more stable over time in treatment colonies (Figure 3-6). The most notable differences in task switching between control and treatment colonies occurred among the active foragers (Figure 3-6). While approximately half of active foragers remained as active foragers between phases in control colonies, only 26.7% remained as active foragers between the pre-exposure Phase 1 and the exposure Phase 2, and 40% remained as active foragers between Phase 2 and the post-exposure Phase 3. A roughly even proportion the active foragers during Phase 1 switched to the intermediate forager group (40%) and the non-forager group (33.3%) during the exposure Phase 2. However, of the active foragers in the exposure Phase 2, 60% shifted to the intermediate forager group and 0% shifted to the non-forager group during the post-exposure Phase 3. Overall, this shows active foragers tended to quit or reduce their foraging activity

during exposure and post-exposure active foragers never quit foraging but most reduced foraging activity.

Table 3-1. Number of bees per task group. Sum of all bees categories in each task group from across all 10 colonies**.** Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure.

Phase	Experimental Group	Task Group		
		Non-forager	Intermediate Forager	Active Forager
Phase 1	Control	203	62	33
	Treatment	224	65	22
Phase 2	Control	252	69	34
	Treatment	285	80	11
Phase 3	Control	320	83	30
	Treatment	341	99	16

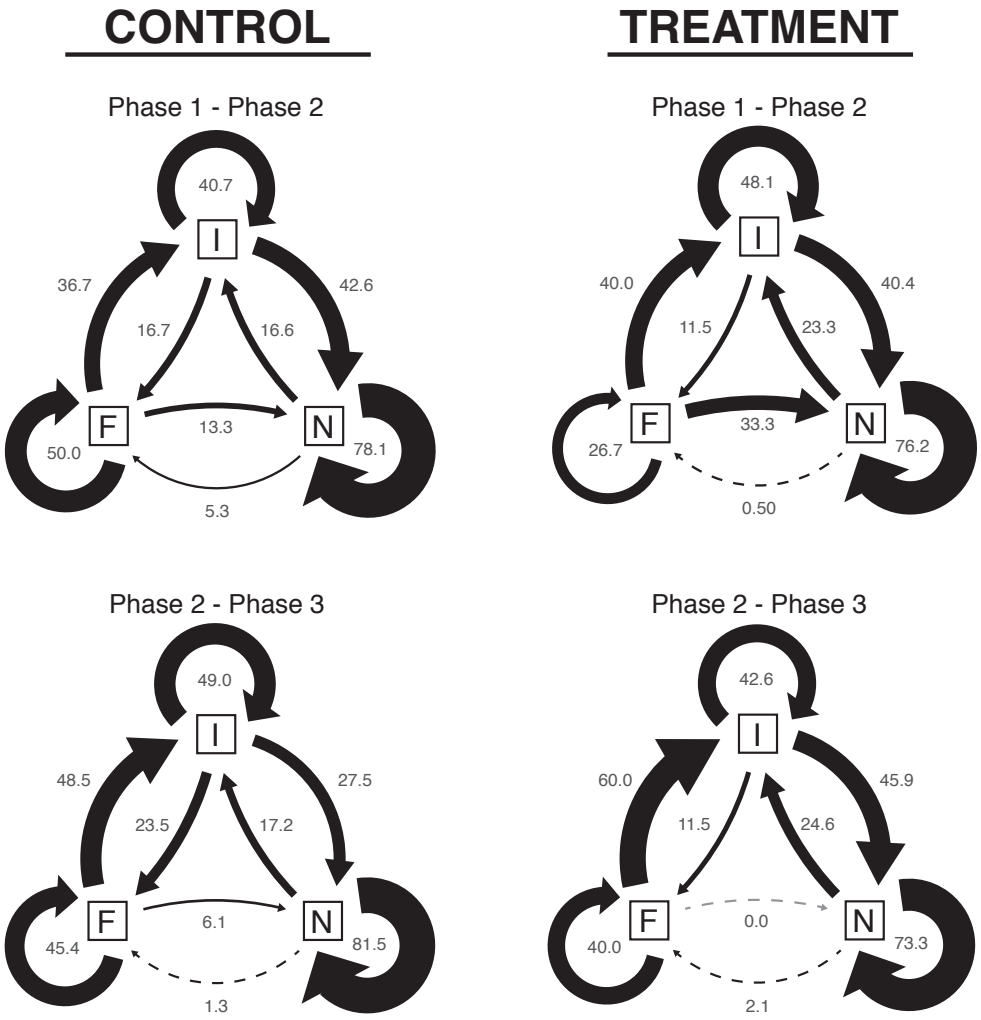

Figure 3-6. Frequencies of task switching between the three bumblebee task groups across experimental phases. Letters in boxes represent task groups: F = active foragers (≥10 foraging bouts over 5 days), I = intermediate foragers (1-9 foraging bouts over 5 days), N = non-forager (0 foraging bouts over 5 days). Numbers show percentages of bees that either remained in the same group or switched to another. Percentages were calculated from only the bees that were present across the pair of phases in question. Percentages within groups have been rounded by the largest remainder method. Arrow thickness is proportional to percentage, except arrows representing <5% have been shown dashed. The 0% switch from forager to non-forager between Phase 2 and Phase 3 is shown in grey.

3.3.3 Locomotor Behaviour of Task Groups in Control Colonies

The following results describe differences in the intranidal locomotor behaviour of different task groups from control colonies only.

The daily median movement speed of all bees in control colonies ranged from 0.14 to 46.44 mm/s (mean = 7.84 mm/s, SD = 4.48 mm/s). Task group accounts for some of this variation (Figure 3-7). There was a significant effect of the interaction between task group (non-forager, intermediate forager or active forager) and phase (Phase 1: pre-exposure, Phase 2: pesticide exposure, Phase 3: post-exposure) on the movement speed of bees in control colonies (LMM $F=4.636$, $p<0.001$). Active foragers were faster than intermediate foragers and non-foragers, and intermediate foragers were faster than non-foragers in each phase (Hypothesis 2a; multiple Tukey post-hoc tests, all $p<0.001$). The speed of intermediate foragers increased significantly between Phase 1 and Phase 3 ($p<0.001$).

Task groups also showed variation in their spatial centrality inside the nest, measured as the distance from the social centroid (DSC)(Figure 3-8). There was a significant effect of the interaction between task and phase on DSC (LMM $F=2.378$, $p=0.050$), however the separate effects of task (LMM $F=97.339$, $p<0.001$) and phase (LMM $F=78.472$, $p<0.001$) were much stronger. Active foragers and intermediate foragers were significantly more distant from the colony social centroid (had higher DSC measurements) than non-foragers in all phases (Hypothesis 2b; multiple Tukey post-hoc tests, all $p<0.001$). There was also a significant overall increase in DSC across phases, with the intermediate group increasing DSC significantly between Phase 2 and Phase 3 ($p<0.001$).

Finally, task groups also had variable home range sizes (Figure 3-9). There was a significant effect of task (LMM $F=73.137$, $p<0.001$) and phase

($F=6.365$, $p=0.002$) on home range size. In each phase, active foragers had significantly larger home ranges than intermediate foragers and non-foragers, and intermediate foragers had larger home ranges than non-foragers (Hypothesis 2c; multiple Tukey post-hoc tests, all $p<0.001$). Unlike the other behavioural measures, home range was significantly smaller during Phase 2 than during Phase 1 ($p=0.022$) and Phase 3 ($p=0.002$).

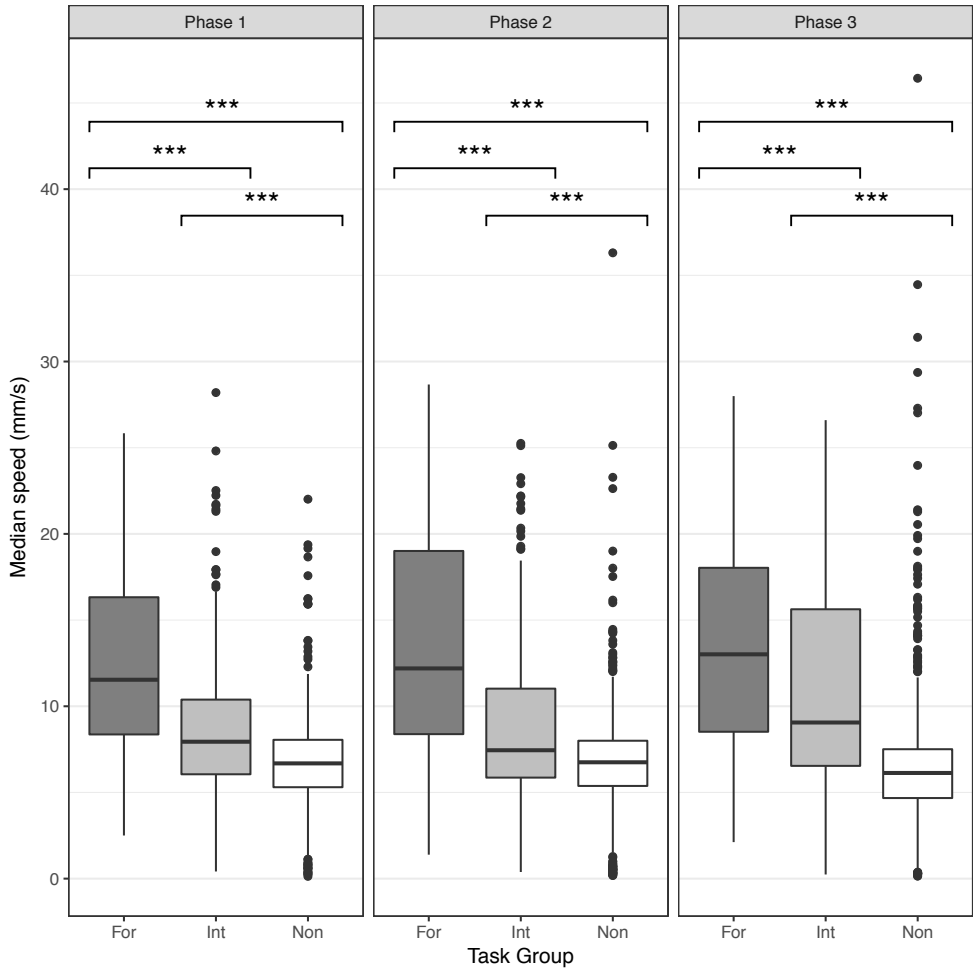

Figure 3-7. Movement speed of task groups in control colonies. The raw intranidal median movement speed measurements of all bees from control colonies in each task group (For = active foragers, Int = intermediate foragers, Non = non-foragers). Separate panels show the movement speed per group in each separate phase. Asterisks between task groups denote statistical differences at $p<0.05$ based on Tukey's post-hoc tests on the output of a linear mixed effects model (see Section 3.3.2).

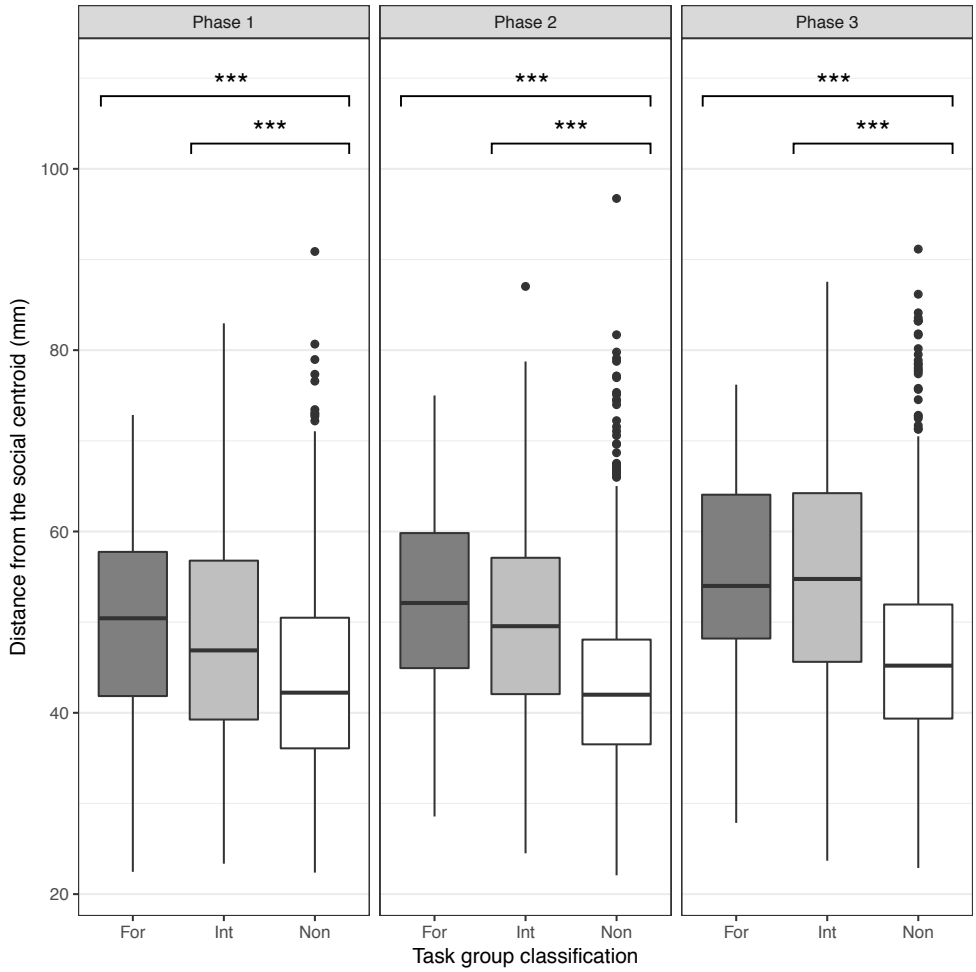

Figure 3-8. Spatial centrality of task groups in control colonies. The raw distance from the social centroid (DSC) measurements of all bees from control colonies in each task group (For = active foragers, Int = intermediate foragers, Non = non-foragers). Separate panels show the DSC per group in each separate phase. Asterisks between task groups denote statistical differences at $p<0.05$ based on Tukey's post-hoc tests on the output of a linear mixed effects model (see Section 3.3.2).

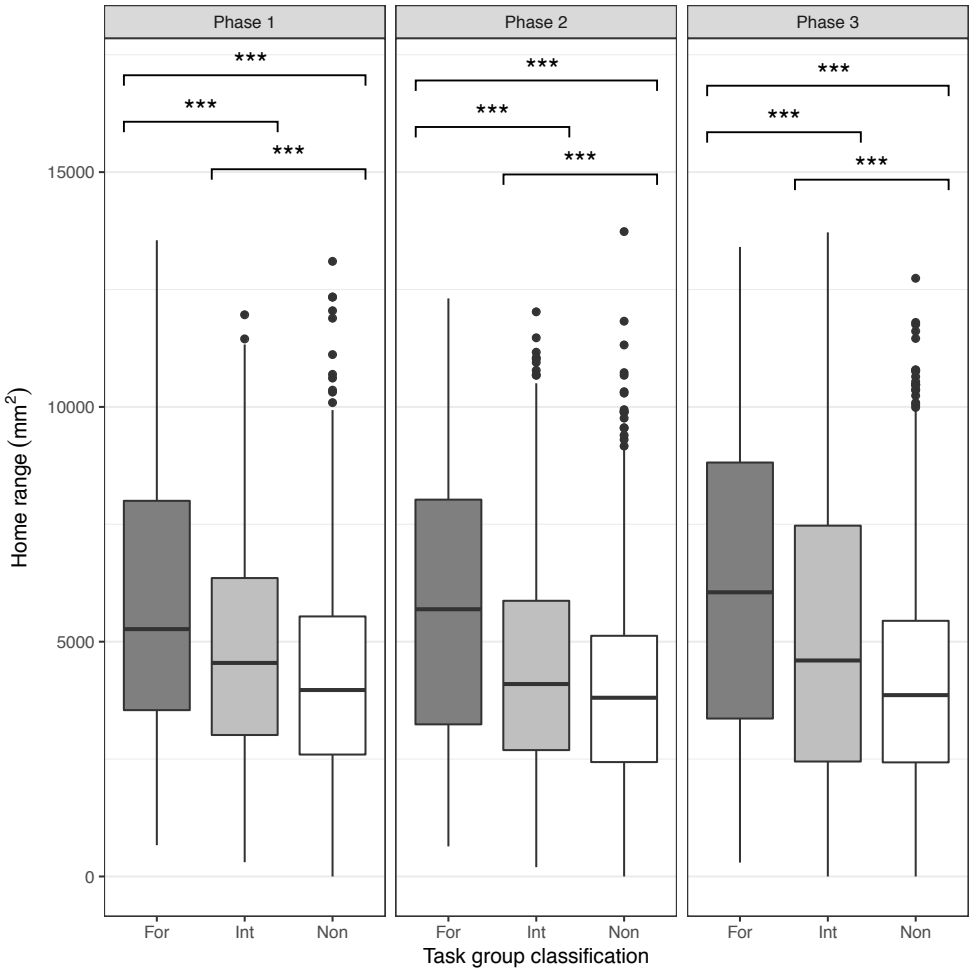

Figure 3-9. Home range of task groups in control colonies. The raw home range measurements (area of 50% minimum convex polygon) of each bee from all control colonies in each task group (For = active foragers, Int = intermediate foragers, Non = non-foragers). Separate panels show the home range per group in each separate phase. Asterisks between task groups denote statistical differences at $p<0.05$ based on Tukey's post-hoc tests on the output of a linear mixed effects model (see Section 3.3.2).

3.3.4 Effects of Pesticide Exposure on Intranidal Movement Speed

The average movement speed of bees was reduced by pesticide exposure and did not fully recovery post-exposure (Figure 3-10). Additionally, the extent of recovery was largely dependent on task group. Overall, there was a significant effect of the three-way interaction between experimental group (control and treatment), phase (Phase 1, Phase 2 and Phase 3) and task group (non-forager, intermediate, forager) on movement speed (LMM $F=3.9208$, $p=0.003$). Specific hypotheses were tested with Tukey's post-hoc contrasts for multiple pairwise comparisons and will be described below.

Movement speed within the treatment group was significantly slower during the pesticide exposure Phase 2 than during the pre-exposure Phase 1 ($p<0.001$). Additionally, the speed of bees in treatment colonies during Phase 2 was significantly slower than in control colonies during Phase 2 ($p<0.001$). Both of these significant comparisons provide strong evidence that the pesticide treatment reduced movement speed (Hypothesis 3a). In the post-exposure Phase 3, movement speed in treatment colonies increased significantly compared to Phase 2 ($p<0.001$), however the speed in Phase 3 remained significantly lower than the speed of control colonies in Phase 3 ($p=0.031$). This suggests that average bumblebee movement speed did not fully recover after the pesticide exposure phase.

The differential effects of pesticide exposure according to phase and task group were also assessed (Figure 3-11). During the pesticide exposure Phase 2, the speed of bees in treatment colonies was significantly slower than bees in control colonies for the forager group, the intermediate group, and the non-foragers group (Tukey post-hoc test, all $p<0.001$), but the size of the effect was not greater for the active foragers (Hypothesis 3b). Recovery of movement speed following pesticide exposure varied according to task group

(Figure 3-11). Notably, the movement speed of active foragers did not recover following pesticide exposure. In the treatment group there was no difference between the speed of bees classified as active foragers in Phase 2 and those in Phase 3 ($p=1.000$). The speed of active foragers in treatment colonies during Phase 3 was also significantly lower than active foragers in control colonies in Phase 3 ($p<0.001$). On the other hand, the movement speed of non-foragers recovered completely during the post-exposure phase. There was no significant difference between the speed of non-foragers in control colonies and treatment colonies during Phase 3 ($p=0.6749$). Intermediate foragers showed some recovery in speed during the post-exposure phase. The movement speed of bees in treatment colonies classified as intermediate foragers during Phase 3 was significantly faster than intermediate foragers in Phase 2 ($p<0.001$), and was no different to the speed of intermediate foragers in Phase 1 ($p=1.000$). However the speed of intermediate foragers in control colonies during Phase 3 was significantly higher than in treatment colonies during recovery ($p<0.001$).

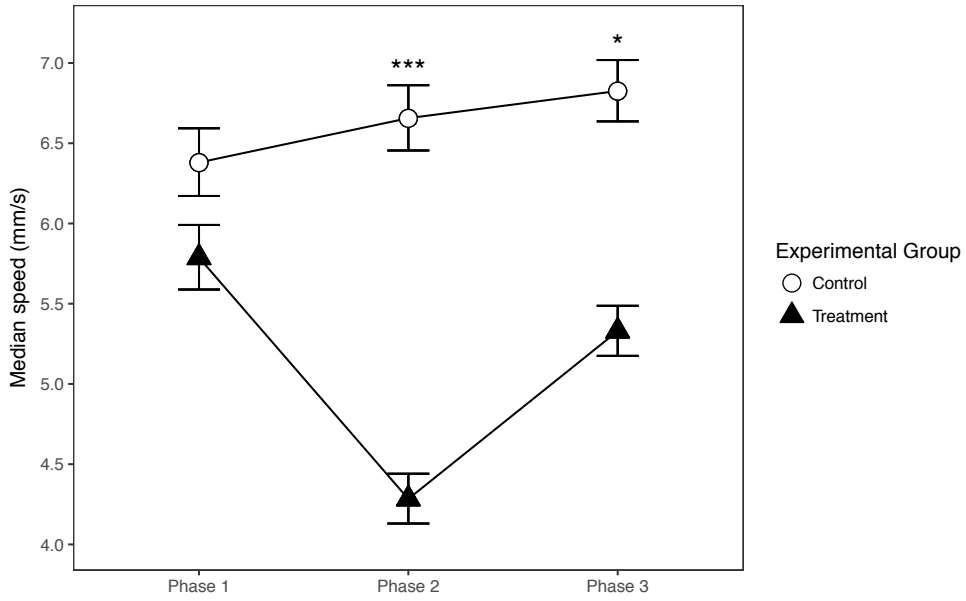

Figure 3-10. Movement speed in bumblebee colonies is reduced by pesticide exposure and it does not fully recover post-exposure. Median speed of bees from control colonies (open circles) and treatment colonies (closed triangles), points show the overall mean estimated from a linear mixed model. Error bars represent 95% confidence intervals. Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure. Asterisks denote statistical differences either between phases (along connecting lines) or between experimental groups (within phases) at $p<0.05$. Only the statistical tests between experimental groups within each phase are shown, for further test results, see Section 3.3.4.

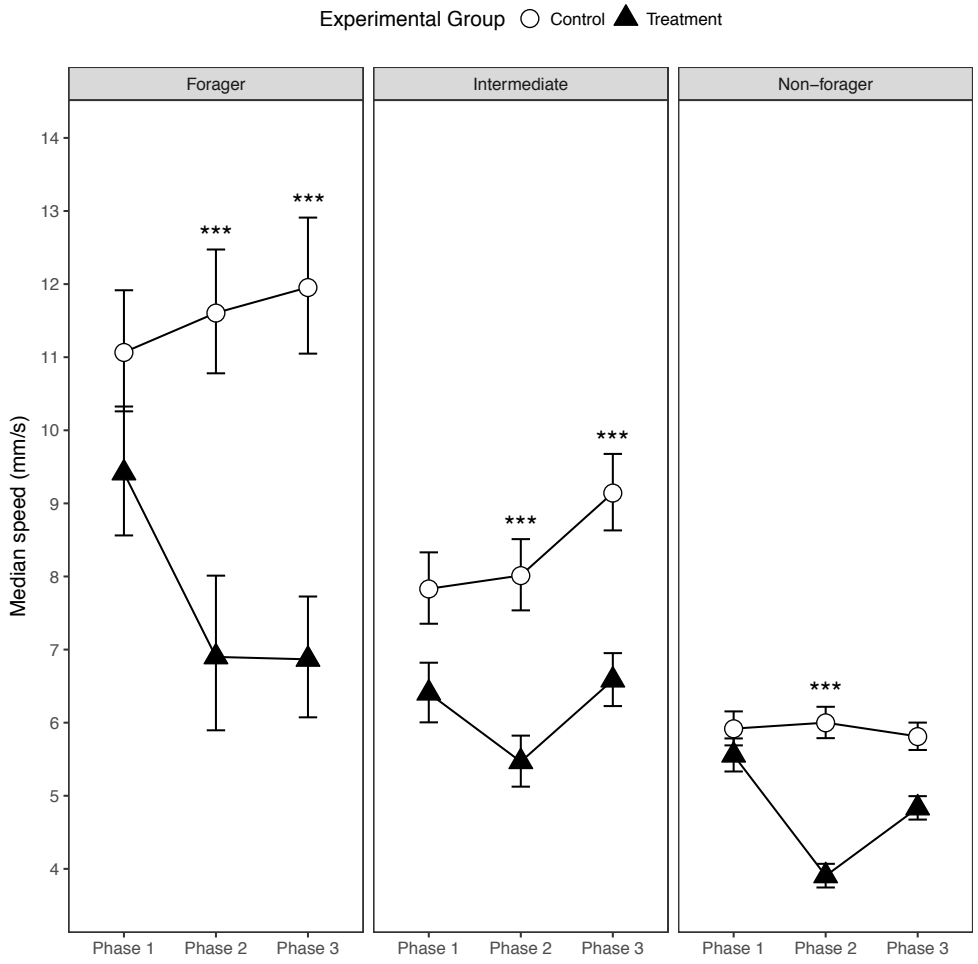

Figure 3-11. Movement speed of all bees reduced during pesticide exposure, but active foragers do not recover post-exposure. Median speed of bees from different task groups from control colonies (open circles) and treatment colonies (closed triangles), shown as means estimated from a linear mixed model. Error bars represent 95% confidence intervals. Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure. Panels contain the results of separate bumblebee task groups defined by foraging activity. Asterisks denote statistical differences between experimental groups within each phase at $p<0.05$. Only the statistical tests between experimental groups within each phase are shown, for further test results, see Section 3.3.4.

3.3.5 Effects of Pesticide Exposure on Intranidal Spatial Centrality

Spatial centrality in the nest was also examined with a LMM, which showed that there was a significant effect of the three-way interaction between experimental group, phase and task on distance from the social centroid (DSC) (LMM $F=3.617$, $p=0.006$). Specific hypotheses were tested with Tukey's post-hoc contrasts and are described below.

There was some evidence that pesticide exposure reduced the DSC of all bees on average from the treatment group during exposure, which was reversed post-exposure (Figure 3-12). Within just the treatment group, there was a significant decrease in DSC between the pre-exposure Phase 1 and the pesticide exposure Phase 2 (Tukey post-hoc test, $p<0.001$), followed by a significant increase between Phase 2 and the post-exposure Phase 3 ($p<0.001$). However, when compared with the control group, there were no significant differences between experimental groups within each phase. These average results for all bees do not provide conclusive evidence that central vs. peripheral nest occupancy is affected; therefore, we cannot reject the null Hypothesis 3c.

With respect to the effect on task groups, the spatial centrality of active foragers was strongly reduced by pesticide exposure, while the effect on other groups was less pronounced (Hypothesis 3d), but all task groups seemed to recover fully post-exposure (Figure 3-13). The DSC of bees classified as active foragers in treatment colonies during Phase 2 was significantly smaller than the DSC of active foragers in control colonies during Phase 2 (Tukey post-hoc test, $p=0.025$). Active foragers also completely recover their normal peripheral nest occupancy after exposure. The DSC of active foragers from treatment colonies increased significantly from Phase 2 to Phase 3 ($p<0.001$),

and the level in Phase 3 was not different from the DSC of active foragers in control colonies during Phase 3 ($p= 0.987$). The effect of pesticide exposure on the DSC of non-foragers and intermediate foragers was less clear (Figure 3-13). Within the treatment group there was a significant reduction in DSC between Phase 1 and Phase 2 for both non-foragers ($p<0.001$) and intermediate foragers ($p<0.001$). This reduction was followed by a significant increase in DSC between Phase 2 and Phase 3 for both non-foragers ($p<0.001$) and intermediate foragers ($p<0.001$). However, as in the results with all bees together, there were no significant differences in DSC between the bees in control and treatment colonies during Phase 1 (non-foragers, $p=0.694$; intermediate foragers, $p=0.719$), Phase 2 (all, $p=1.000$), or Phase 3 (all, $p=1.000$).

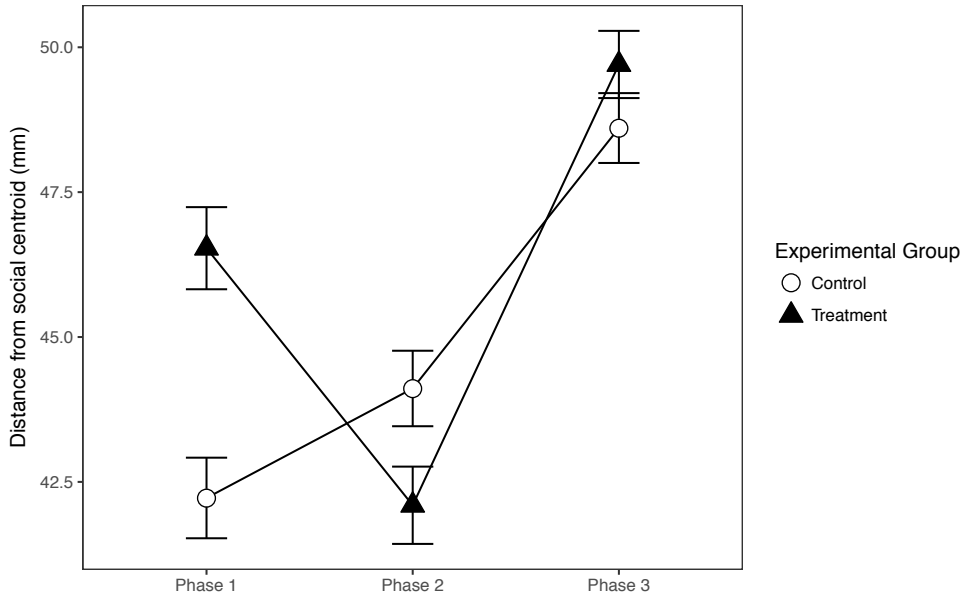

Figure 3-12. Distance from the social centroid appears to decrease during pesticide exposure, also recovers post-exposure. Points show the estimated mean spatial centrality of bees in control colonies (open circles) and pesticide treatment colonies (closed triangles), measured as distance from the social centroid and estimated by a linear mixed model (see text for details). Error bars represent 95% confidence intervals. Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure. There were no statistical differences between experimental groups within each phase at $p<0.05$. Statistical tests of other pairwise comparisons are not shown, see Section 3.3.5.

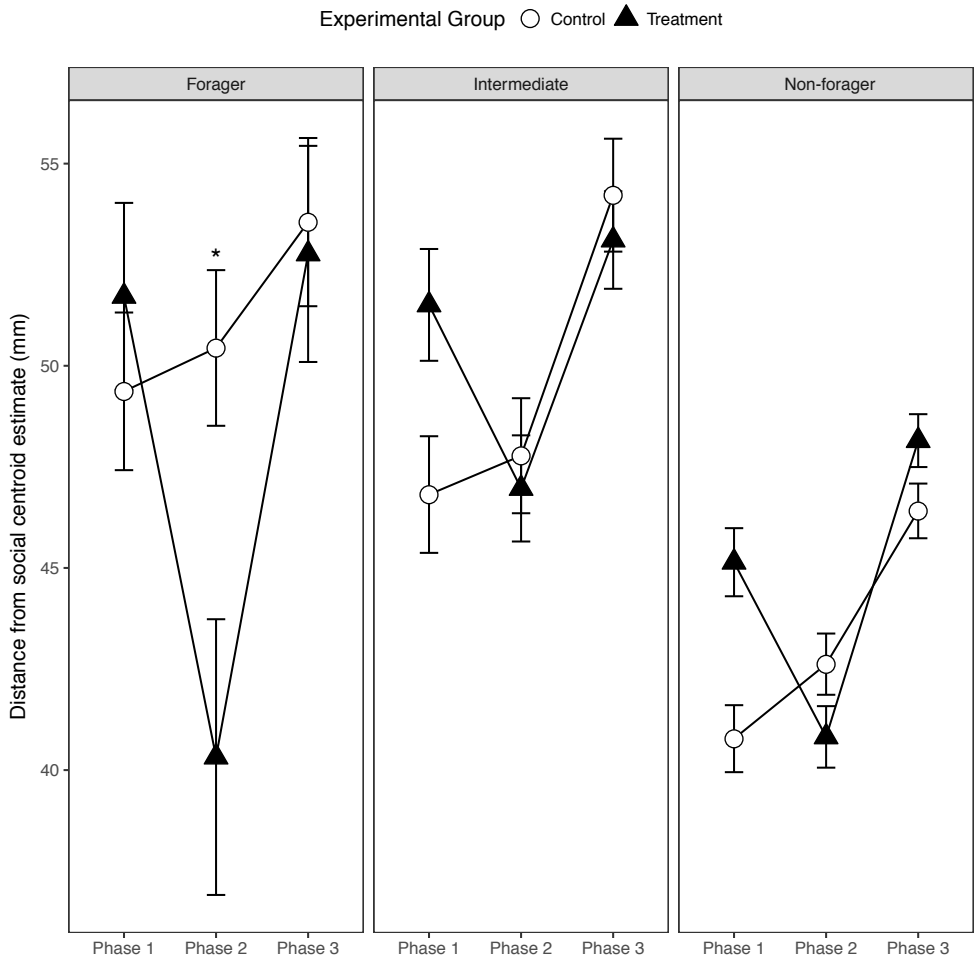

Figure 3-13. Distance from the social centroid is most strongly decreased for active foragers during pesticide exposure, all task groups recover post-exposure. Points show the estimated mean spatial centrality of bees in control colonies (open circles) and pesticide treatment colonies (closed triangles), measured as distance from the social centroid and estimated by a linear mixed model (see text for details). Error bars represent 95% confidence intervals. Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure. Panels contain the results of separate bumblebee task groups defined by foraging activity. Asterisks denote statistical differences between experimental groups within each phase at $p<0.05$. Only the statistical tests between experimental groups within each phase are shown, for further test results, see Section 3.3.5.

3.3.6 Effects of Pesticide Exposure on Intranidal Home Range

The results of modelling the factors affecting home range size showed that the three-way interaction between experimental group, phase and task group on home range size was not significant (LMM $F=1.4300$, $p=0.221$). In the model that best described the data there was a significant effect of the interaction between group and phase on home range size (LMM $F=126.39$, $p<0.001$), plus a significant effect of task on home range size (LMM $F=121.91$, $p<0.001$). This simplified model was used to conduct multiple comparisons of means using Tukey post-hoc contrasts as in previous sections.

Considering the home range measurements of all bees, pesticide exposure appeared to cause bees in treatment colonies to occupy smaller areas of the nest (Figure 3-14). Within the treatment group, home range decreased significantly between the pre-exposure Phase 1 and the pesticide exposure Phase 2 ($p<0.001$). Following pesticide exposure, home range size showed a significant increase between Phase 2 and the post-exposure Phase 3 ($p<0.001$). Additionally, the home range of bees in treatment colonies during the pesticide exposure Phase 2 was significantly smaller than control colonies during Phase 2 ($p<0.001$), providing strong evidence that this decrease was attributable to the pesticide exposure. Furthermore, there were no significant differences between control and treatment home range sizes within Phase 1 or Phase 3, suggesting normal home range size recovered on average after exposure.

The effect of pesticide exposure on home range size followed a very similar pattern within each task group, which was reflected in the no-effect result of the group*phase*task interaction above (Figure 3-15). Bees classified as non-foragers in treatment colonies during Phase 2 had significantly smaller home ranges than non-foragers in control colonies during the Phase 2 ($p<0.001$).

This reduction in home range area can be strongly attributed to pesticide exposure because there was no significant difference in the home range of non-foragers in control colonies and treatment colonies during Phase 1 ($p=0.930$), plus there was no change in home range in control colonies between Phase 1 and Phase 2 ($p=0.999$). The home range of non-foragers also recovered completely following pesticide exposure (Figure 3-15). During Phase 3 there was no difference between the home range of non-foragers in control and treatment colonies ($p=1.000$). The other task groups in treatment colonies showed a significantly smaller home range size during Phase 2 compared to both Phase 1 (forager, $p<0.001$; intermediate, $p<0.001$) and Phase 3 (intermediate, $p<0.001$). This reduction in home range size, followed by apparent recovery is consistent with the effect of pesticide exposure seen in non-foragers. However, there were no significant differences between active foragers in control and treatment colonies or between intermediate foragers in control and treatment colonies during Phase 1 (active foragers, $p=1.000$; intermediate foragers, $p=0.804$), Phase 2 (active foragers, $p=0.195$; intermediate foragers, $p=0.581$), or Phase 3 (active foragers, $p=0.986$; intermediate foragers, $p=1.000$).

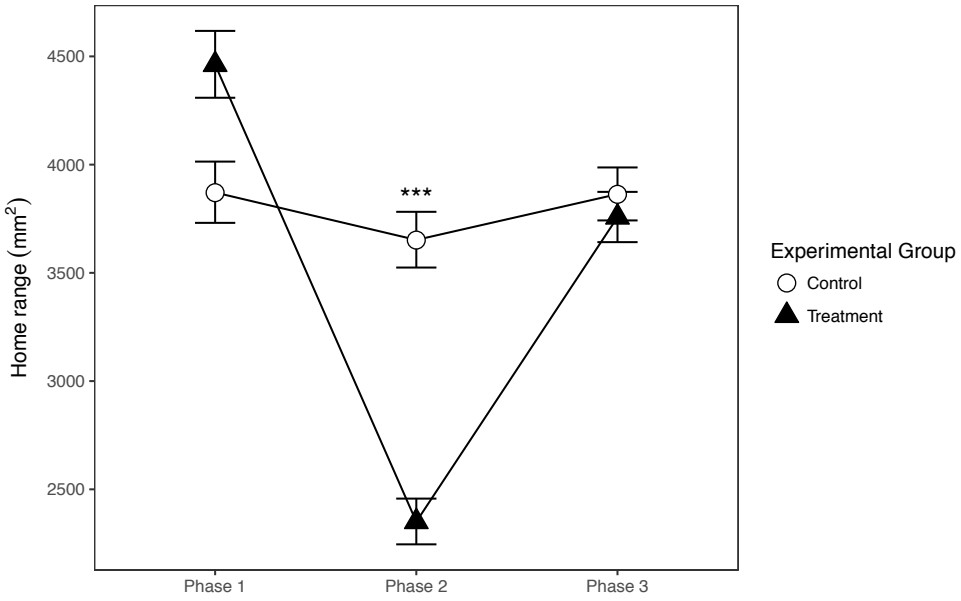

Figure 3-14. Home range inside the nest decreased by pesticide exposure, followed by recovery post-exposure. Points show the mean estimated home range of bees in control colonies (open circles) and pesticide treatment colonies (closed triangles), measured as the area of a minimum convex polygon enclosing 50% of each bee's spatial occupancy inside the nest. Means estimated by a linear mixed model (see text for details). Error bars represent 95% confidence intervals. Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure. Asterisks denote statistical differences at $p<0.05$. Only the statistical tests between experimental groups within each phase are shown, for further test results, see Section 3.3.6.

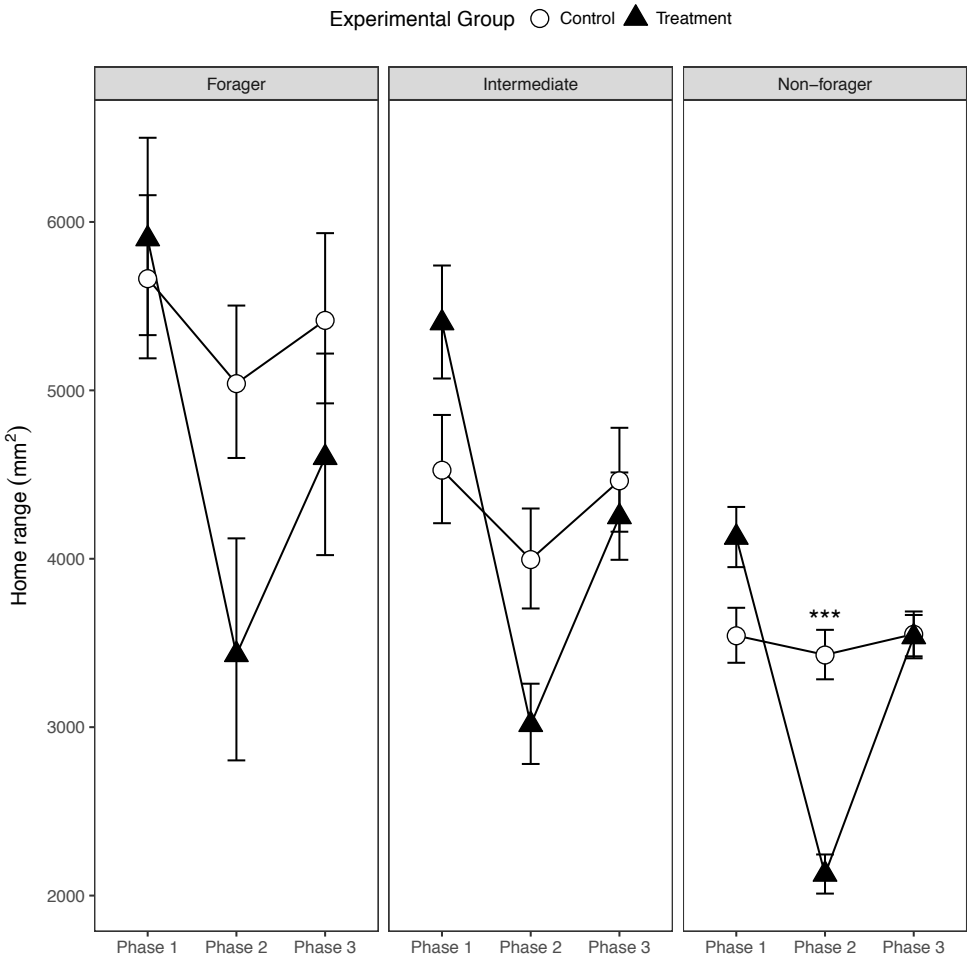

Figure 3-15. Home range inside the nest decreased similarly for each task group during pesticide exposure, followed by recovery in all task groups post-exposure. Points show the estimated mean home range of bees in control colonies (open circles) and pesticide treatment colonies (closed triangles), measured as the area of a minimum convex polygon enclosing 50% of each bee's spatial occupancy inside the nest. Means estimated by a linear mixed model (see text for details). Error bars represent 95% confidence intervals. Pesticide treatment was tested in phases: Phase 1 = pre-exposure, Phase 2 = exposure, Phase 3 = post-exposure. Panels contain the results of separate bumblebee task groups defined by foraging activity. Asterisks denote statistical differences at $p<0.05$. Only the statistical tests between experimental groups within each phase are shown, for further test results, see Section 3.3.6.

3.4 Discussion

Inter-individual variation combined with behavioural plasticity in social insects generates flexible systems of task allocation across a range of social and environmental contexts, allowing colonies to adapt to novel disruptions. A great deal of evidence shows how neonicotinoid pesticides can disrupt individual behaviour, and colony performance, but the interdependent nature of the individual and the colony has been overlooked in pesticide research. This study took the novel approach of tracking the behaviour of entire queenright bumblebee colonies during pesticide exposure. This holistic, high-throughput approach has shown that socially regulated task allocation can lead to differential effects of pesticide exposure on the behaviour of bees in different task groups. The comparisons between colony-wide foraging effort, individual foraging activity, and the behaviour of different groups inside the nest during exposure all contribute to the conclusion that monitoring one component of individual or colony-level behaviour in isolation is not enough to fully understand the sublethal effects of pesticide exposure on social bee colonies.

The overall foraging activity of treatment colonies was not significantly affected by pesticide exposure, but there was some evidence of shifts in individual behaviour. Neither the total number of foraging bouts, nor the total number of foragers (including active foragers and intermediate foragers) showed a significant change during exposure. However, there did appear to show a decreasing trend in some colonies, plus there was some evidence that there were fewer active foragers during pesticide exposure. Due to the high variation between colonies there may not have been enough power to detect a subtle effect on overall foraging activity. It is possible that if the level of replication was increased, then an effect on naturally variable levels of

foraging activity may have been detected at this level of imidacloprid exposure. Yet, there was a detectable decrease in the foraging activity per individual (number of bouts per forager) during pesticide exposure, suggesting again that there were trends of reduced foraging activity. Given these results it is not possible to suggest there was a colony-level response to low food intake, which would have been characterised by an increase in the total number of foragers, a stable number of bouts and a decrease in the number of bouts per forager.

At the individual-level, it seems the trends of decreasing foraging rate during pesticide exposure may have been due to an increase in the amount of time all foragers spent inside the nest between foraging bouts. Several studies have reported reductions in foraging activity outside the nest during pesticide exposure (e.g. Gill and Raine, 2014; Lämsä et al., 2018; Schneider et al., 2012). In this case however, the time that all foragers from treatment colonies spent *outside* the nest during foraging bouts was remarkably consistent with control colonies. In contrast, the time spent *inside* the nest between foraging bouts (the inter-bout duration) was significantly longer during pesticide treatment. Longer inter-bout duration correlates with the decreased intranidal movement speed observed during exposure. The differences in the effects on forager behaviour inside the nest and outside the nest could suggest flight may be less affected than walking, or there may be some other social mechanism that keeps the forager engaged inside, such as an increase in the time spent engaged in social interactions (Nicolis et al., 2005). This finding highlights the importance of testing multiple components of behaviour during pesticide risk assessment. Studies such as Morandin and Winston (2003) that do not detect any effects of neonicotinoid exposure regimes on foraging bouts may have overlooked effects on forager behaviour inside the nest.

It is important to note the limitations of the laboratory foraging set-up when drawing conclusions from these results. Foraging bees in this experiment only had to walk down a 30 cm tube and fly approximately 70 cm (as the crow flies) to reach the feeder. Wild bumblebee foragers regularly fly hundreds of meters per bout to visit distant flower patches, where they will have to fly between many flowers searching for nectar and pollen (Woodgate et al., 2016). Therefore, the single feeder is not likely to present a realistic foraging challenge for bumblebees. Nevertheless, the decreasing trends suggested here are concerning. If the laboratory foraging regime is considered 'easy' for bees, then neonicotinoids at this dose are likely to cause greater harm to foraging in free-flying colonies (as shown by Gill and Raine, 2014 and Gill et al., 2012).

Classification of worker task groups by their foraging effort is a simple technique that can define biologically meaningful groups in bumblebees (Yerushalmi et al., 2006). Despite the relatively simple foraging task, dividing the bees into tasks groups based on foraging activity ('active foragers', 'intermediate foragers', or 'non-foragers') produced groups with significantly different locomotor behaviours that matched well with traits of bumblebees task groups described by previous work (Dornhaus and Chittka, 2001; Free, 1955b; Goulson et al., 2002; Jandt and Dornhaus, 2009; Jandt et al., 2009). Namely, individuals classified as active foragers were typically larger bees that maintained high movement speeds within the nest, and occupied large areas of the nest, including the periphery. In contrast, individuals classified as non-foragers (based on a lack of observed foraging effort), were smaller, had slower movement speeds, and occupied smaller and more central areas of the nest. The intermediate forager group displayed patterns of locomotor behaviour in the middle of this spectrum and therefore describes some of the

flexibility in bumblebee task allocation system. The similarity between these task groups and those described by previous studies also supports the power of automated techniques to accurately describe behaviour.

On average, the movement speed of bumblebees in queenright colonies decreased significantly during pesticide exposure and did not recover in the week after pesticide exposure ceased. Yet this average result masked the markedly different effects observed within each task group. The response of active foragers (≥10 bouts per phase) followed the same patterns as the colony average; movement speed decreased significantly during exposure and did not recover post-exposure [of the bees classified as active foragers in the post-exposure phase, 40% were also classified as active foragers during the exposure phase (Figure 3-6)]. The movement speed of non-foragers (0 bouts per phase) also showed a similar significant decrease during exposure but the speed of these non-foraging bees recovered during the post-exposure phase (to a level that was not significantly different from the control group in the post-exposure phase). The recovery of non-forager movement speed was in contrast to the colony average response, which did not recover. Previous work has shown that individually isolated bumblebees (*B. terrestris*) exposed to imidacloprid in nectar at 98 ppb (much higher than 'field-realistic') for 3 days, significantly increased their activity (proportion of observations when the individual bee was in motion) the day after exposure ceased (Cresswell et al., 2013). The same study measured residues in the bodies of individual bees and estimated that bumblebees clear neonicotinoids from their system after 48h (Cresswell et al., 2013). The results of the present study suggest that the recovery of individually isolated bumblebees shown by Cresswell et al. (2013) best describes the recovery of non-foraging workers within queenright colonies. Active foragers, on the other hand, show no recovery at all, thus

appear to be much less able to detoxify neonicotinoids after a 7-day pulsed exposure. Intermediate foragers (1-10 bouts per phase, i.e. up to 2 bouts per hour over 5 days) form a much less distinct group than the other two groups defined here and any conclusions or comparisons drawn from their behaviour should be treated with caution. The movement speed of intermediate foragers also decreased during exposure. Intermediate foragers recovered their movement speed to the same level as before pesticide exposure, but at a level that was significantly less than intermediate foragers in the control group.

The distance from the social centroid (DSC) of active foragers (≥10 bouts per phase) also showed a different pattern of behaviour over time compared to the colony average changes over time. The effect on the colony average DSC during pesticides was not as clear as the effect on movement speed. Within the treatment group, there was strong evidence for a decrease in DSC (less peripheral and more central spatial occupancy) during pesticide exposure, followed by a complete recovery post-exposure. However, this pattern over time in treatment colonies overlapped with the gradual upward trend in control colony DSC over time, with no differences between treatment groups at any phase. This colony average result might suggest that DSC is simply too variable between colonies and may not represent an important metric of bumblebee behaviour. Yet, there was strong evidence of a decrease in DSC in the forager task group during pesticide exposure, followed by a full recovery. The recovery of spatial occupancy shown by active foragers is in contrast to the total lack of recovery seen in active forager movement speed, suggesting different social or physiological mechanisms affect these two behaviours in different ways. Non-foragers and intermediate foragers showed almost exactly the same pattern of DSC changes over time as the colony average.

The final metric of locomotor behaviour inside the nest was home range size. The colony average showed clear evidence that neonicotinoid exposure significantly reduced intranidal home range, but that this space-use behaviour recovered completely post-exposure. However, the non-foragers were the only group that had a significantly smaller home range size during pesticide exposure than the control group. The non-foragers also showed a full recovery. There was some evidence for a decrease in home range size in the forager group and intermediate group, but these were not significantly different from those in control colonies in any phase. The mean home range of non-foragers during pesticide exposure could be approximated to a 4.5×4.5 cm square (within an 18×10 cm nest box). This significant effect on non-foragers, combined with some evidence of a tendency for more central nest occupancy during exposure could amplify the effect that the spatial distribution of larval feeding has on adult size ranges (if non-foragers are engaged in brood care). Peripheral larvae receive less food and do not grow as large as central larvae, which results in differences in adult worker size (Couvillon and Dornhaus, 2009). Any shifts from the natural size range in bumblebee colonies could further impact colony-level task allocation (Jandt and Dornhaus, 2014)

In summary, bumblebees within queenright colonies experience differential toxic effects of neonicotinoid exposure depending on their level of foraging activity (or foraging inactivity). Active foragers displayed slow average movement speeds and their spatial occupancy shifted toward the nest centre. At the other end of the scale, non-foragers also moved more slowly on average during pesticide exposure, and occupied significantly smaller nest regions as well. The current study also found differential task-related recovery after pesticide exposure. Non-foragers recovered completely in all behavioural

metrics following a pulse of pesticide exposure, whereas forager movement speed did not recover, but forager central-peripheral nest occupancy did. The natural behaviour of successful returning foragers is crucial for the organisation of foraging effort across the colony (Dornhaus and Chittka, 2001) and disruptions to their movement patterns could have consequences on foraging-related information flow (see Chapter 4). If returning foragers that have been exposed to neonicotinoids are not able to run throughout the nest distributing pheromones and increasing social contacts (Dornhaus and Chittka, 2001), then they may not be able to sufficiently activate other workers to leave the nest and forage themselves. The outcome of forager intranidal behavioural impairment on the colony was not determined in this case. Colonies foraging in agricultural land exposed to a pulse of neonicotinoids in mass-flowering crops could suffer disruptions in food intake and the organisation of work if foragers are unable to recover. Although there was no evidence of precocious foraging here, the severe and long-lasting effects of exposure on both active and intermediate forager behaviour could cause colonies in the field to reallocate extra workers to foraging, which could end up depleting the colony workforce as the chances of worker losses increase (Gill et al., 2012; Perry et al., 2015)

This study shows that pesticide testing and risk assessment protocols must monitor bee behaviour within the social context of the colony, or they risk overlooking sublethal effects on collective behaviour that could have serious consequences for colony health.

Chapter 4

Effects of Neonicotinoids on Bumblebee Social Interaction Networks

4.1 Introduction

Complex systems are made up of many interacting components and tend to display global-level attributes that cannot be explained simply by the sum of the attributes of the individual components. In other words complex systems exhibit non-linear scaling (Bar-Yam, 1997b). Network theory proposes that the global-level attributes of complex systems are determined by the topological structure and dynamics of the network of interactions that exists between the system's components (Barabási, 2016). This network-based approach to describing complex systems is becoming increasingly popular with behavioural ecologists wishing to understand how the behaviour of individuals scale up to generate the complex patterns of social organisation seen in many animal groups, from primates to insects (Croft et al., 2008). The pressures of natural selection are thought to have affected the evolution of complex biological networks to the extent that they can be considered to be adaptive (Bonabeau, 1998). The evolved resilience and flexibility of these networks, in the face of variable environments, is of great interest in terms of

our understanding of the natural world, but also in the design of complex man-made systems such as computer networks.

Social insect colonies are remarkable examples of complex biological systems; they are composed of many independent individuals that exhibit relatively simple behaviour, and yet colonies exhibit collective behaviour far exceeds the behavioural capacity of the individual (Oster and Wilson, 1978). Examples of complex collective behaviour in social insects include the architecture of spiral cooling vents in termite mounds (Collins, 1979), the hub-and-spoke arrangement of foraging raids of army ants (*Eciton burchelli*) (Franks and Fletcher, 1983), and the group-level decision making of optimal nest sites in honeybees (*Apis mellifera*) (Seeley et al., 2012) and rock ants (*Temnothorax albipennis*) (Robinson et al., 2009a). There is no centralised control in social insects; instead these patterns are self-organising and emerge as a result of distributed interacting individuals responding to local social and environmental information (Camazine et al., 2001; Sumpter, 2006). The self-organisation approach allows us to conceptualise the mechanisms behind collective behaviour, but the key to understanding the emergence of self-organising complexity lies is in describing the various ways individuals respond to local information and how social interactions lead to the flow of information across the group (Sumpter, 2006). Interactions and behavioural responses between pairs of social insects occur frequently in colonies and can appear to have very simple actions and consequences. For example, the exchange of food during trophallaxis until satiation (Sendova-Franks et al., 2010) or the winner/loser outcomes of dominance contests in paper wasps (West-Eberhard, 1969). But how do pair-wise interactions scale up to produce colony-level complexity as suggested by the concept of self-organisation? By taking a network approach to analysing such interactions we can enhance our

understanding of how the behaviours of individuals scale to generate the behaviours of the colony (Charbonneau et al., 2013; Croft et al., 2008; Naug, 2015). When the intricate web of interactions in a colony is modelled as a network we can see that the structure and dynamics of the networks themselves have global-level properties that influence key colony processes such as the regulation of task partitioning (Baracchi and Cini, 2014; Mersch, 2016; Mersch et al., 2013), the efficient dissemination of food (Sendova-Franks et al., 2010), the transmission of disease (Naug, 2008; Otterstatter and Thomson, 2007), and the formation of dominance hierarchies (Shimoji et al., 2014; Shizuka and Mcdonald, 2015).

The network-level property underlying all of these colony behaviours is flow. In social insect colonies this represents the flow of information, resources and disease that pass between individuals during interactions. Given the opposing pressures of supporting the transmission of beneficial information and resources, while limiting the spread of disease, flow in social insects networks is seen as an adaptive trait. This view is supported by multiple studies that have found network flow to be rapid in the short term and within small clusters of individuals, but constrained over longer time scales across the whole colony (Blonder and Dornhaus, 2011; Pinter-Wollman et al., 2011; Richardson et al., 2017). Processes of flow are also resilient to colony perturbations, such as the loss of individuals (Jeanson, 2012; Naug, 2008). Information flow also confers colonies with great flexibility and resilience in response to changes in the environment or colony demographics (Naug, 2008).

A particularly important issue that could benefit from viewing colonies as complex systems is understanding the effects of pesticide exposure on colony function in social bees (Heimbach et al., 2017; Potts et al., 2010b). Social bees in agricultural landscapes face a range of anthropogenic stressors and the non-

target exposure of bees to systemic neonicotinoid pesticides is thought to one of the most significant, but this explanation remains controversial (Alkassab and Kirchner, 2017; Goulson et al., 2015). Part of the reason for this controversy lies in the mixed results of experiments that expose either individuals or colonies to field-realistic doses of neonicotinoids (Alkassab and Kirchner, 2016; El Hassani et al., 2008; Piiroinen et al., 2016; Rundlöf et al., 2015; Woodcock et al., 2017). We are unable to explain why we see effects at some levels but not others; this is partly because we do not understand how pesticide-induced changes in individual behaviour scale up to produce sub-optimal colonies. This study will take complexity theory approach to understand the effects of neonicotinoids on the emergent properties of pollinating bee colonies by employing analytic techniques from social network theory to describe changes in patterns of social interactions.

4.1.1 Social Interactions in Bumblebees

Bumblebees (*Bombus terrestris*) make an ideal model system for investigating the potential impact of neonicotinoid exposure on social interaction networks. First of all, bumblebees are important managed and wild pollinators that are at risk of exposure to neonicotinoids from treated crops, and it is vital to understand the level of the risk posed by neonicotinoids to bees. It is also possible to maintain bumblebee colonies in laboratory conditions where all individuals in the colony can be tracked and manipulated, and pesticide exposure regimes can be properly controlled. Importantly, bumblebees also engage in contact-based social interactions that contribute to colony-level organisation. For example, contact interactions between bumblebee foragers and their nest mates contribute to the regulation of foraging activity (Dornhaus and Chittka, 2004). When a successful bumblebee forager returns

to the nest she must find a suitable nectar pot where she can unload her crop of nectar before she can leave to forage again. During this inter-bout period, foragers also perform an erratic "zig-zag" run throughout the nest, periodically contacting nest mates and fanning their wings (Dornhaus and Chittka, 2001; Renner and Nieh, 2008). Forager contacts have been shown to increase the probability of a contacted nest mate leaving the nest to forage herself, leading to the suggestion that this zig-zag run could serve to increase contact with nest mates and therefore increase forager recruitment (Renner and Nieh, 2008). Contact interactions can also affect disease transmission in bumblebee colonies. Otterstatter and Thomson (2007) found that the rate of contact interactions between an individual bumblebee (*Bombus impatiens*) and her infected nest mates was the only significant predictor of infection from the gut parasite *Crithidia bombi*. Additionally, at the colony-level, contact networks with a high network density (the proportion of observed edges relative to all possible edges) had higher rates of disease transmission. An individual's contact network position plus the structure of the network therefore influence disease transmission dynamics. Bumblebee contact networks can be said to face the same constraints of maintaining efficient information flow while restricting disease spread as other social insects interaction networks.

Van Honk and Hogeweg (1981) described antennation in bumblebee colonies as an interaction where two bees "stop walking, antennate each other for a moment and then one of the workers retreats and walks away." Retreating is considered submissive because the queen will rarely retreat from antennation interactions and workers that do not retreat tend to initiate aggression towards nest mates later in the colony cycle (van Honk and Hogeweg, 1981). Ultimately, antennation interactions form a social hierarchy

among workers in which there is a distinct high-dominance group of 'elite' workers that rarely retreat. These dominant bees have a high rate of antennation interactions, their position in the hierarchy approaches the alpha position of the queen and they are the most likely to lay their own male eggs during the competition phase (van Doorn and Heringa, 1986; van Honk and Hogeweg, 1981). Hogeweg and Hesper (1983) simulated developing bumblebee colonies and showed that such interactions could generate a self-organised dominance hierarchy resembling real colonies. Initially identical bees randomly engage in antennation interactions that affect individual dominance status via a self-reinforcing mechanism; less dominant bees more likely to retreat from future interactions, while more dominant bees become more likely to 'win' future encounters. The outcome was a social hierarchy that affected individual space-use, task allocation and reproductive potential. Taken together, these results demonstrate how simple local interactions in bumblebee colonies are significant elements in the self-organisation of bumblebee social organisation.

4.1.2 Neonicotinoids and the Potential for Social Disruption in Bumblebees

Many previous studies have reported negative effects of neonicotinoid exposure on individual-level locomotor behaviour in social bees. Honeybees (*Apis mellifera*) show dose-dependent responses to neonicotinoid exposure ranging from hyperactivity, loss of postural control and increased time spent flying at low acute doses (Alkassab and Kirchner, 2018; Lambin et al., 2001; Tosi and Nieh, 2017; Tosi et al., 2017; Williamson et al., 2014), to reduced rate of movement, reduced flight duration and distance, and reduced the ability to climb at higher chronic doses (Teeters et al., 2012; Tosi and Nieh,

2017; Tosi et al., 2017). Bumblebees have also been shown to suffer reduced rate of movement at high doses of imidacloprid (Cresswell et al., 2013). Additionally, Chapter 3 showed that chronic exposure to imidacloprid (10 ppb) significantly decreased movement speed and caused bees to occupy smaller, more central regions of the nest. Taken together, these effects on locomotor behaviour could disrupt the mechanics of contact-based interactions in bee colonies and thus restrict the extent to which individuals are able to interact. Colonies of ants that exhibit spatial segregation of workers are characterised by higher rates of interactions within segregated groups than between groups (Mersch et al., 2013). This segregation of interactions and interaction partners could affect colony functioning by limiting in information flow (Blonder and Dornhaus, 2011), which is important in flexible task allocation.

Another important component of social interactions that could be affected by the behavioural deficits described in Chapter 3 is the rate of social interactions. Interaction rate in animal groups is strongly influenced by individual movement speed and the density of individuals (Adler and Gordon, 1992; Backen et al., 2000; Pacala et al., 1996). Reduced movement speed could lead to reduced interaction rates. Although some ants maintain constant interaction rates over a range of densities by clustering and increasing local density (Gordon, 1999; Gordon et al., 1993). Some degree of spatial clustering was seen in Chapter 3, which could suggest a behavioural response to the effects of neonicotinoid exposure on locomotor function.

Neonicotinoid exposure has been shown to reduce the number of waggle dance circuits performed by honeybee (*A. mellifera*) foragers (Eiri and Nieh, 2012). However, the effects of pesticide of bumblebee forager interactions have not been tested. Chapter 3 showed that active foragers and non-foragers were

affected differently during exposure to pesticides in terms of locomotor behaviour. Active foragers displayed greatly reduced movement speeds inside the nest during pesticide exposure and did not recover post-exposure. The movement speed of non-foragers was also affected during exposure, but non-foragers made a complete recovery post-exposure. These differential behavioural effects could disrupt the mechanics of forager behaviour inside the nest and the resulting contact interaction patterns between bees engaged in foraging and bees engaged in nest work. Given the importance of forager contact interactions in the regulation of foraging activity (Dornhaus and Chittka, 2001; Renner and Nieh, 2008), these between task group interactions are a crucial organisational component of bumblebees colonies that could be disrupted by neonicotinoid exposure.

An important functional consequence of disrupted social interaction during pesticide exposure could be a knock-on effect to colony-wide information flow. Slow movement speeds and small spatial displacement among ants, for example, produced rates of information flow that were faster in the short term and slower in the long term than a null gas particle diffusion model (Blonder and Dornhaus, 2011). Exposure to pesticides in Chapter 3 reduced the movement speed and the spatial displacement of bees; therefore, these behavioural effects could change the dynamics of information flow in bumblebee colonies.

4.1.3 Aims and Hypotheses

The primary aim of the current work was to record social interactions in *B. terrestris* colonies and test the robustness of the structure and function of the interaction networks during a pesticide exposure experiment. First, the structure of social networks was described by measuring the diversity of

interaction partners and the interaction rate of bumblebee interaction networks. Next, network structure was further described by quantifying mixing patterns between categorical bumblebee task groups (foragers and non-foragers). The changes in these network features during the experiment were used to reveal how colony growth, exposure to pesticides and recovery from exposure interact to affect social organisation. Any effects of pesticide exposure on networks structure or interaction patterns could have knock-on effects for network function and ultimately colony performance. Finally, the functional properties of bumblebee interaction networks were measured in terms of the capacity of the network to support information flow. A disruption to colony information flow induced by pesticide exposure could have serious consequences for crucial social processes such as flexible task allocation in colonies in agricultural landscapes.

4.1.3.1 Hypothesis 1: Pesticide exposure will decrease the extent of social mixing

Contact-based social interactions between individuals could be disrupted by pesticide-induced changes to the movement patterns of bees inside the colony. Therefore, the first hypothesis was: pesticide exposure will reduce the extent to which all colony members interact (Hypothesis 1a). This study also aimed to test the resilience of bumblebee interaction patterns; therefore, the second part to the first hypothesis concerns the post-exposure behavioural recovery of bumblebees. According to Chapter 3, bumblebee locomotor behaviour recovered during the week post-exposure, therefore Hypothesis 1b was: the extent to which all colony member interact will recover during the post-exposure phase.

4.1.3.2 Hypothesis 2: Pesticide exposure will decrease social interaction rate

Movement speed decreased during pesticide exposure (Chapter 3), which leads to the first part of the second hypothesis: interaction rate will decrease during the exposure phase (Hypothesis 2a). Once again, this study aimed to test network resilience, the movement speed of bees recovered post-exposure (Chapter 3), leading to the second part of the second hypothesis: the interaction rates will recover after exposure (Hypothesis 2b). However, following on from Hypothesis 2a, bumblebees also clustered towards the centre of the nest during the pesticide exposure, which could act to increase interaction rates by adjusting local density. This possibility that bumblebees may cluster to increase interaction rates during exposure leads to the null hypothesis: there will be no effect of exposure on interaction rate.

4.1.3.3 Hypothesis 3: Pesticide exposure will decrease interactions between foragers and non-foragers

The first task-group hypothesis is: task-group (foragers and non-foragers) mixing will decrease during pesticide exposure (Hypothesis 3a). The exposure to pesticides in Chapter 3 also caused foragers to spend significantly longer inside the nest between foraging bouts. This increased inter-bout duration could act to regulate the interactions each forager experiences during its time inside the nest. This could explain a scenario in which there was no evidence for Hypothesis 3a. Given forager movement speed did not recover post-pesticide exposure (Chapter 3), the second task-group hypothesis was: there will be no recovery post-exposure of disruptions to task group mixing patterns caused during pesticide exposure (Hypothesis 3b).

4.1.3.4 Hypothesis 4: Pesticide exposure will reduce information flow

Contingent on any observed effects on interactions, the fourth hypothesis was: pesticide exposure will decrease colony information flow (Hypothesis 4a). According to the behavioural recovery of most bees during the post-exposure phase of Chapter 3, the second part of the fifth hypothesis was: information flow will recover post-exposure (Hypothesis 4b).

4.2 Methods

The experimental methodology of this study was the same as in Chapter 3, but will be described here in brief. A total of 10 bumblebee colonies began the experiment with a queen plus 50 workers each. All individuals were marked with unique barcode-like tags from a 16-bit version of the BEEtag video tracking system (Crall et al., 2015). Colonies were kept in artificial nest boxes in the laboratory, and were able to forage for nectar *ad libitum* in an enclosed foraging arena. Nest boxes were fitted with red lights to illuminate the colonies for video recording inside the nest. Of the 10 colonies, half were assigned to the pesticide treatment group, while the other half were not treated with any pesticide and act as a control group. The pesticide treatment schedule followed a simple baseline–experiment–recovery design. The schedule consisted of 5 days pre-exposure, 7 days exposure, followed by 7 days post-exposure (total = 19 days). During the pesticide exposure phase treatment colonies were supplied with nectar in the foraging arena containing the neonicotinoid pesticide imidacloprid at a concentration of 10 $\mu g kg^{-1}$ (10 ppb), while control colonies were supplied with untreated nectar. Colonies were filmed inside the nest and at the external nectar feeder for 1 hour every day. Video data analysis was constrained to three 5-day phases: all 5 days

pre-exposure (day 1-5 = Phase 1), the last 5 days of exposure (day 8-12 = Phase 2), and the last 5 days of post-exposure (day 15-19 = Phase 3).

4.2.1 Social Interactions and Network Analysis

Social interactions were detected automatically from the video-tracking trajectories of individual bees inside colonies. Acquisition of video-tracking trajectories is described in Chapter 2. The methodological details of the two different interaction detection techniques are also described in Chapter 2, but their application, with respect to answering the hypotheses outlined above, will be discussed here. The first interaction type was based on physical proximity between pairs of trajectories and was implemented in such a way as to record instances of physical contact between pairs of bees. Interactions based on physical proximity, hereafter referred to as proximity interactions, describe a contact-based social encounter that can inform individuals bees of colony density and foraging activity (Renner and Nieh, 2008), or transmit disease (Otterstatter and Thomson, 2007), for example. The second interaction type was based on the relative geometric positions of individually modelled antennal ranges. The antennal range of each bee was modelled as a polygon approximating an annular sector, termed the 'interaction zone', which was connected to the individual's video tracking trajectory. In this way, the interaction zone approximated the position of an individual's antennae. The interactions zones were used to record approximations of antennation interactions when a pair of interaction zones overlapped and the pair of bees they described were facing head-to-head (for full details, see Chapter 2). These approximate antennation interactions, hereafter referred to as head-to-head (HTH) interactions, were more specific than proximity interactions and may describe antennation interactions related to dominance,

which can influence worker reproduction and task allocation (van Doorn and Heringa, 1986; van Honk and Hogeweg, 1981).

Interactions detected via the two automated techniques described above were used to construct separate networks for each colony during each 1-hour observation period. Network nodes were individual bees, while edges were either proximity interactions or head-to-head interactions. Edges were undirected and weighted by the frequency of interactions between each dyad (pair of interacting nodes). Proximity interactions networks and HTH networks will be compared and contrasted to describe the basic structure of bumblebee interaction networks and address Hypothesis 1 and Hypothesis 2. Due to the well-described link between contact interactions and forager information flow (Dornhaus and Chittka, 2001; Dornhaus and Chittka, 2004; Renner and Nieh, 2008), Hypothesis 3 and Hypothesis 4 will be specifically addressed with reference to proximity networks, although the patterns observed in HTH networks will be mentioned for completeness.

The collective patterns of colony-wide interactions were described by recording the network metrics mean degree and mean strength, described below. The most basic property of a node in a network is the number of edges connected to it, this is known as the node's degree. In a social network, the degree of an individual (node) is a measure of the number unique relations that individual has with other members of the social group. The average number of edges per node is a network-level metric called mean degree k. For an undirected network, mean degree is a measure of social differentiation. In time-aggregated networks of bumblebee interactions (1 hour observation window), mean degree represents the number of unique individuals each bee interacts with per hour on average, which is informative of the level of social

mixing within the colony in that time; therefore mean degree will be used to address Hypothesis 1.

Mean degree is a measure that considers edges as binary, i.e. either the relation is present (denoted by a value of 1), or the relation is absent (denoted by a value of 0). Edges in a network can also have a weight associated with them, which reflects a measure of the strength of each relation and can be denoted by values greater than 1. Incorporating edge weights into the analysis of animal social networks is important when observed networks represent only a sample of the true relationships that exist within a social group (Lusseau et al., 2008; Whitehead, 2008). This is because the binary state of unweighted edges may not describe the true presence or absence of a relation within a group; the relation may not have been observed or may have been mistakenly identified. In this case, edge weights in bumblebee social networks were determined by the number of interactions that occurred between each pair during the 1-hour observation period (e.g. if bee #1 and bee #2 are observed interacting three times during an hour, the value of the weighted edge connecting them will be 3). The weighted degree of a node, also know as node strength, is the number of unique social interaction partners, weighted by the total number of interactions with each (e.g. if bee #1 and bee #2 are connected by a weighted edge with a value of 3, and bee #1 and bee #3 are connected by a weighted edge with a value of 2, the node strength of bee #1 is equal to 5). As with unweighted degree, the average node strength (mean strength) across all nodes is a network-level measure of the mean interaction frequency and will be used to address Hypothesis 2. These network-level metrics will be taken from independent colonies (tracked over time), which will be analysed using conventional statistics (Croft et al., 2011).

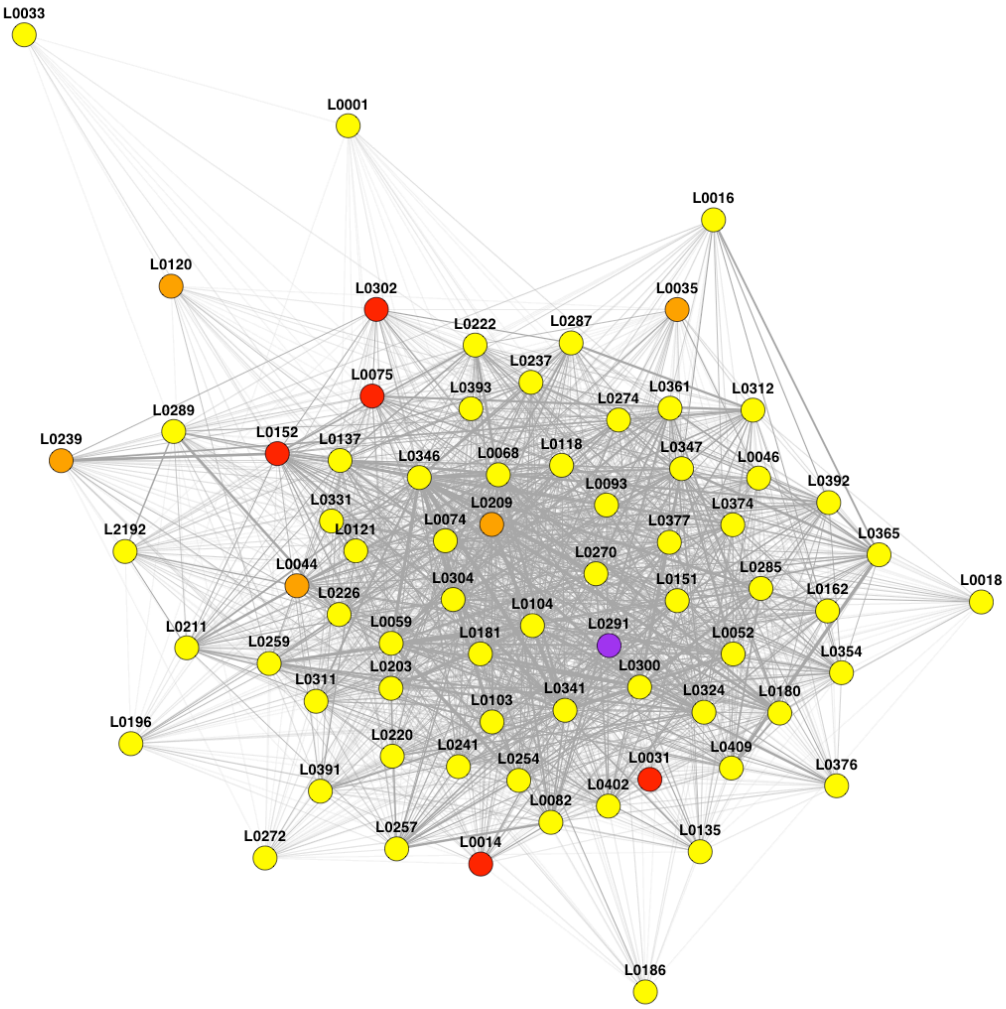

Figure 4-1. Example bumblebee proximity interaction network. Network diagram showing proximity interactions (edges = grey lines) between bumblebees (nodes = coloured circles) in a representative control colony (Colony L). Edge thickness is proportional to interaction frequency. Nodes are labelled with unique bee ID numbers. The purple node is the queen; the red nodes are workers who completed more than one foraging bout in the 1-hour observation period; the orange nodes are workers who completed one foraging bout; the yellow nodes are workers who who did not forage.

4.2.2 Task Group Mixing Patterns

The patterns of mixing between task groups within bumblebee colony social networks were quantified for each 1-hour observation period across all colonies. Two task group categories were considered here: 'foragers' completed at least one foraging bout per observation period; 'non-foragers' did not leave the nest to forage during the observation period (n.b. these daily task group definitions are different to the by-phase (5-day) task group definitions of Chapter 3). Mixing patterns within and between categories of network nodes can be represented as an $m \times m$ mixing matrix **e**, where m is the number of categorical groups in the network (Croft et al., 2008; Newman, 2003). The elements of the matrix e_{ij} represent the proportion of network edges that occur between vertices of category i and vertices of category j. The diagonal elements of the matrix, e_{ii}, represent the proportion of interactions that occur within groups, whereas the off-diagonal elements describe the proportion of edges that occur between different groups. When interactions are undirected, the edges between groups are divided equally between the upper triangle and the lower triangle of the matrix, i.e. the matrix is symmetric and $e_{ij} = e_{ji}$, which establishes the following sum rules

$$\sum_{ij} e_{ij} = 1, \qquad \sum_{j} e_{ij} = a_i, \qquad \sum_{i} e_{ij} = b_j \,,$$

where a and b are the row and column sums, respectively, of the mixing matrix **e**. In undirected networks $a = b$. The value a_i represents the proportion of edges that begin at vertices of category i and end at all other categories including i. Therefore the elements of a inform us of the true proportion of edges that involve each category.

A mixing matrix can be used to measure the extent of mixing within and between categories. Newman (2003) developed a technique, based on the mixing matrix, to quantify the tendency for networks to assort according to node categories, i.e. to quantify the extent of *within*-category mixing. The result is Newman's assortivity coefficient r, which incorporates a null model into the calculation to provide an estimate of the proportion of edges that occur within categories above what would be expected if the network edges were randomised with respect to category – referred to as the "excess assortment" (Newman, 2003). This estimate of assortivity ranges from perfectly assorted (all edges occur within categories) to perfectly disassorted (all edges occur between different categories). In this case, it was hypothesised that mixing patterns *between* groups may be affected by the experimental pesticide exposure, therefore Newman's r was adapted to record the extent of *between*-category mixing, and simplified for two m=2 categories.

For the current study there were m=2 categories: foragers (F) and non-foragers (N). An example mixing matrix can be represented as

$$e = \begin{pmatrix} 0.10 & 0.05 \\ 0.05 & 0.80 \end{pmatrix},$$

where proportion of edges that within the forager category $f = 0.1$, the proportion of edges within the non-forager category $n = 0.8$, and the proportion of edges that are between categories $c = 0.05 + 0.05 = 1 - f - n$. The expected proportion of edges for f, n and c are calculated based on the row sums and column sums as in a standard contingency table,

$$\mathrm{E}(f) = a_1 b_1 = {a_1}^2,$$

$$\mathrm{E}(n) = a_2 b_2 = {a_2}^2,$$

$$\mathrm{E}(c) = (a_1 b_2) \; + \; (a_2 b_1) \; = \; 2(a_1 a_2),$$

where a and b are the row sums and the columns sums of, respectively, of the mixing matrix **e**. These expected proportions of edges within and between groups form the null expectation of assortivity. Therefore, the simplified coefficient of the extent of "excess" mixing between two categories is given by

$$s = c - \mathrm{E}(c) \big/ 1 - \mathrm{E}(c),$$

where the denominator is included to normalise values of s (see Newman, 2003). If there is complete inter-category mixing (i.e. all edges occur between categories) then this adapted coefficient $s = 1$, and if there is no excess inter-category mixing above the expected null values then $s = 0$. If the network is perfectly assorted (i.e. all interactions occur within categories) and there are no inter-category edges, then s is negative in the general range $-1 \leq s < 0$. The minimum value of s (see Newman, 2003) is given by

$$s_{min} = -\mathrm{E}(c) \Big/ (1 - \mathrm{E}(c)).$$

Once a value for s has been calculated, it is necessary to determine if the value differs statistically from zero. A jackknife simulation procedure can be used to estimate the variance of a measured value of s (Croft et al., 2008; Newman, 2003). Resampling the M edges of the network via a jackknife procedure is appropriate because each edge can be treated as an independent contributor to the matrix **e.** An approximation of the variance of a measured value of s is given by

$$\sigma_s^2 \approx \sum_{i=1}^{M} \left(s_i - s\right)^2,$$

where s_i is the calculation of s from the resampled network with the i^{th} edge removed. The calculated values of s from multiple colonies over time will reveal the extent to which bumblebee task groups are segregated or integrated. Significantly positive values of the coefficient s would reveal that foragers and non-foragers interact more than expected, while significant negative values would show that interactions occur mostly within task groups, suggesting significant segregation between forager and non-foragers. Additionally, calculated values of s were compared between control and treatment colonies to test for a disruptive effect of pesticide exposure on bumblebee colony task group association patterns.

4.2.3 Network Flow

Bumblebee social network dynamics were investigated by measuring processes of flow. Network flow refers to the pattern and rate of transmission of a disease, resource, or unit of information across a social group via the interactions between individuals. Flow dynamics can be tracked over time when the interactions in the network are explicitly modelled as time-ordered (Blonder et al., 2012). Time-ordered bumblebee proximity networks were used to simulate the spread of a hypothetical unit of information across the colony (Section 4.2.3.1). These simulations were used to describe the rate of flow generated by contact-based transmission and to test the hypothesis that pesticide exposure reduces the potential for information flow across the temporal networks of pesticide-treated colonies. The rate of flow was considered in terms of 'growth' of the proportion of individuals reached by the hypothetical information over time and was modelled by a mathematical

growth function (Section 4.2.3.2). The growth model of cumulative bumblebee information flow was used to describe the dynamics of the system and to compare pesticide treated colonies with controls.

4.2.3.1 Informed-Uniformed Model

The potential for information flow was simulated according to the framework of a deterministic susceptible-infected (SI) model (Anderson and May, 1992). SI models were developed to simulate the spread of disease in human social networks, but have recently been used to simulate the flow of disease, resources and information across temporal networks in social insect groups (Blonder and Dornhaus, 2011; Gernat et al., 2018; Quevillon et al., 2015; Sendova-Franks et al., 2010). In an SI model, all individuals in the networks exist in one of two states, "susceptible" or "infected". When a susceptible individual interacts with an infected individual, they become "infected". When the flow of a hypothetical unit of information is simulated in an empirical temporal network, the results describe the potential for the time-ordered interactions to support flow dynamics (Blonder et al., 2012).

This study developed a simple SI model in the R programming environment (R Core Team, 2016) to simulate the potential spread of information brought back into the nest by successful foragers. The model inputs were a time-ordered network of automatically detected social interactions, an initial "seed" of the simulated information, the identities of all foragers and the times of foraging bouts synchronised to the time-ordered network. The two states "infected" and "susceptible" are equivalent to the terms "informed" and "uniformed" therefore this model used the latter pair while discussing information flow. The initial seed of the simulated information was the first forager to complete a foraging bout, i.e. the first bee

to leave the nest, visit the feeder and return to the nest during the observation period (for full details of recording foraging bouts, see Chapter 2). This condition ensured the seed was an active forager and could introduce foraging-related information into the colony. To set the seed for the simulation, the first active forager was set to the informed state at the time they returned to the colony. This model was deterministic, i.e. uninformed bees that interact with an informed bee become informed themselves. This deterministic model simulates the empirical upper bound of the rate of information flow in bumblebee temporal networks (Blonder and Dornhaus, 2011; Gernat et al., 2018). The model also considers every subsequent successful forager (an individual that has completed a foraging bout within the observation period) as informed, even if they have not previously interacted with another informed individual. This condition includes the possibility that all active foragers could continue to introduce foraging-related information to the colony and influence foraging recruitment. The output of the model was a sequence of the identities of newly informed individuals over time. The model was run on the network of each daily 1-hour observation period.

4.2.3.2 Curve Fitting

The aim of modelling the simulated potential for information flow across temporal networks was to quantify flow dynamics and test the hypothesis that pesticide exposure would quantitatively disrupt flow (Hypothesis 4). To quantify flow dynamics, the rate of increase in the proportion of informed individuals (P) as a function of time (t), $dP(t)/dt$, was modelled by an analytic growth model (model selection described below). Such a model takes the inputs of an initial proportion of informed individuals P_0 (i.e. a single

forager bee divided by the total number of bees that could be informed), and a carrying capacity K (i.e. all individuals informed P=1). The parameters of this growth model can then be used to compare the dynamics of information flow between colonies and to quantify the impact of pesticide exposure on information flow.

Several models commonly used to describe growth in biological systems were tested to see how well they fit the information flow data generated by the SI model described above (see Table 4-1). Only growth models with an asymptote at 1, corresponding to all individuals in the colony informed, were considered as candidates. The solutions of the growth models, P(t), were fitted to the data and the resulting models were compared based on goodness-of-fit and model parsimony. Curve fitting was achieved via non-linear least squares with the R function 'nls'. The Bayesian Information Criterion (BIC) was used to compare models. This model comparison technique was chosen because it assesses model fit while including a penalty for the number of parameters (increasing the number of parameters can lead to overfitting). Low BIC scores were favourable. The candidate models had different numbers of parameters, which made comparison by methods such as mean square error unsuitable. The candidate functions considered were the Verhulst logistic, Turner's generic growth, the Weibull, hyperbolic regenerative growth, Von Bertalanffy's, Richard's, the Gompertz, and the hyper-Gompertz. The solutions of these equations are shown in Table 4-1. Given the maximum proportion of informed individuals (the carrying capacity) was a known constant across all curves (K=1) and that the initial proportion of informed individuals (the y-intercept, P_0) was also a known constant for each individual curve, candidate models with these parameters were substituted with the constants before curve fitting. This step reduces the number of

parameters in these models thus leading to compatible BIC scores. The generic logistic growth function (Turner's generic growth) and Richard's growth function could not be compared against the other functions because of problems with model convergence. Model fitting with the 'nls' function is very sensitive to the selection of initial parameter estimates and for these two models this limitation made them unusable in this context (where 119 separate models were fitted). The remaining growth functions were fitted to the information flow curve of each observation period (see Figure 4-6) and the model BIC score was determined in each case.

Table 4-1. Candidate growth models compared during information flow curve fitting. Where applicable, parameters are: P_0, the y-intercept; K, the upper asymptote; and additional shape parameters r, a, b, n and γ, with nomenclature inherited from source. Lowest BIC is a count for each growth function of the number of times that growth function scored the lowest BIC score compared to the other growth function for each colony information flow curve (total = 119).

Function Name	**Solution**	**Lowest BIC**
Verhulst Logistic	$P(t) = \frac{KP_0}{(K - P_0)e^{-rt} + P_0}$	0/119
Weibull	$P(t) = K - (K - P_0)e^{-rt^{\alpha}}$	9/119
Hyperbolic Regenerative Growth	$P(t) = \frac{K(t + a)^n}{b + (t + a)^n}$	34/119
Von Bertalanffy	$P(t) = K\left[1 - \left\{1 - \left(\frac{P_0}{K}\right)^{1/3}\right\}e^{-\left(rt/3K^{1/3}\right)}\right]^3$	0/119
Gompertz	$P(t) = K\left(\frac{P_0}{K}\right)^{e^{-rt}}$	0/119
Hyper-Gompertz	$P(t) = K \times exp\left[-\left\{(\gamma - 1)rt + \left[\ln\left(\frac{K}{P_0}\right)\right]^{1-\gamma}\right\}^{1/(1-\gamma)}\right]$	76/119

4.2.4 Statistical Analyses

Statistical analyses were conducted in the R programming environment (version 3.3.0; R Core Team, 2016). Linear mixed models (LMM) were used to test the effects of social interaction type (proximity and HTH), experimental group (control and treatment) and phase (Phase 1, Phase 2 and Phase 3) on continuous response variables. Generalised linear mixed models (GLMM) were used to test the effects of the same fixed effects on count data, using a negative binomial error distribution. Mixed models were used to help account for the non-independence of repeated measures within phases, which each consisted of five daily samples per colony. Colony ID was included as a random factor to account for the non-independence of daily samples by defining each colony data point as the mean of five days per phase. These 5-day means per colony per phase were considered to be independent as all phases are separated by 2-day gaps in sampling. Models were constructed using the R package 'lme4' (Bates et al., 2015). Models were simplified according to backward stepwise elimination to identify significant predictors of the response variable; predictors were eliminated when they did not improve model fit at critical $p<0.05$. Several response variables were transformed before model fitting to improve the normality of residuals and to reduce heteroscedasticity in the relationship between model residuals and fitted values. The sum of weights (sum contacts/number of interactions) was square root transformed and the hyper-Gompertz r parameter was log transformed. Any estimated parameter values from models of transformed response variables were untransformed accordingly.

4.3 Results

Automated proximity interaction detection recorded 602,253 interactions across all colonies and days in the control group and 632,682 interactions in the treatment group. For HTH interactions there were 191,396 interactions recorded in control colonies and 206,476 detected in treatment colonies. There were fewer recorded HTH interactions due to the fact that the automated detection conditions were more specific than for proximity interactions.

4.3.1 Network Size

Although not specifically addressing the main hypotheses, network size influences other properties of the network (James et al., 2009); therefore, the size of the experimental bumblebee networks will be discussed here.

Social interactions were recorded automatically from video tracking trajectories and each colony network forms a single connected component (e.g. Figure 4-1); therefore, the number of nodes in each network (network size) was equal to the number of bees detected via video tracking (the number of tracked bees) per colony. In an attempt to standardise network size, all colonies began the experiment with 50 marked workers plus a marked queen. However, several factors affected network size throughout the experiment, including the emergence of new bees, the death of old bees, video tracking performance and trajectory data pre-processing (Figure 4-2).

The number of marked adult bees increased over time in all colonies, with the exception of Colony G and Colony F (Figure 4-2). These two colonies were monitored in parallel as a control (G) and treatment (F) pair and both suffered high adult mortality and very low rates of brood development as a result of their advanced colony-level maturity at the beginning of the experiment. Additionally, both colonies were marked by the emergence of

gynes and the queen from Colony F died on Day 17. For these reasons, these two colonies were not considered to be representative bumblebee colonies in the ergonomic growth phase, but instead represent colonies that are nearing the end of their lifecycle. This late stage in bumblebee colony development is associated with changes to interaction patterns and colony-level social organisation (van Doorn and Heringa, 1986); therefore, these two colonies were excluded from any further analyses. In the remaining eight colonies, the total number of tracked bees was less than the number of tagged bees at each time point (for a full description of video tracking performance see Chapter 2), but still increased over time as colonies grew. There was no evidence for an effect of the pesticide exposure Phase 2 on either the number of tagged bees or the number of tracked bees. This was shown by the non-significant effect of the interaction between experimental group (control or treatment) and phase (baseline Phase 1, exposure Phase 2, post-exposure Phase 3) on both the number of tagged bees (GLMM group*phase, χ^2=4.031, p=0.133) and the number of tracked bees (GLMM group*phase, χ^2=3.762, p=0.152). The absence of an effect of pesticide exposure on the number of tagged bees is not surprising for two reasons. Firstly, the dose of imidacloprid used is considered sub-lethal and should not cause increased mortality directly (see Chapter 1). Second, the adults that emerged during Phase 2 and Phase 3 had already pupated before Phase 2 and therefore could not have been exposed to any dietary imidacloprid. Although there was no overall effect of pesticide exposure on the number of tracked bees, Colony I appeared to show a decrease in tracked bees during pesticide exposure (see Figure 4-2). There were fewer tracked bees present in the data from Colony I during the exposure Phase 2 (52.4±0.5 SE) than during the baseline Phase 1 (61.4±0.4 SE). The primary driver of this decline was behavioural; a relatively large

number of bees remained outside the nest in the foraging arena during the pesticide exposure phase (data not shown).

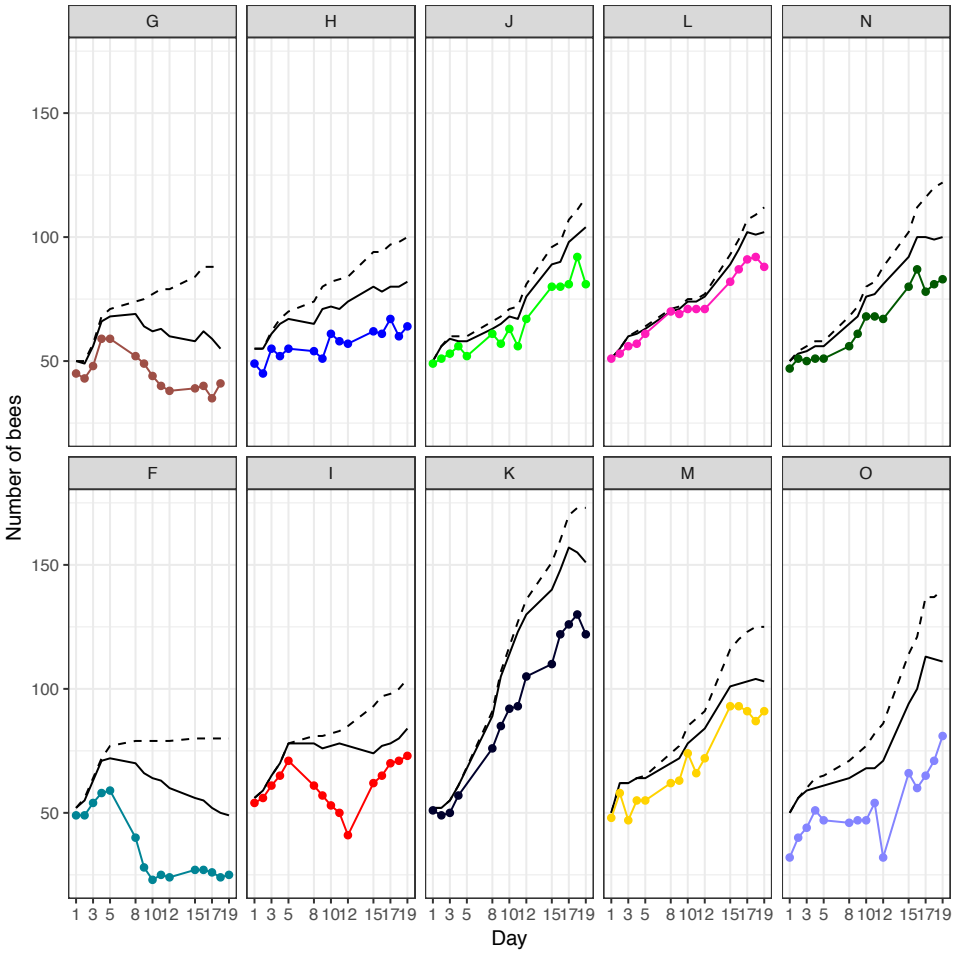

Figure 4-2. Colony growth and the number of tracked bees Daily records of colony growth plus the number of bees detected by video tracking. Colonies in the control group are in the first row; the treatment group is in the second row. Each column contains paired experimental colonies that were monitored in parallel. Dashed lines show the total number of tagged bees in each colony. Solid lines show the number of tagged bees minus those that died. Coloured lines show the number of unique bees tracked by video tracking.

4.3.2 Social Mixing

In proximity networks, both control and treatment colonies showed an overall increase in mean degree between the beginning and end of the experiment, but mean degree appears to decrease in treatment colonies during the pesticide exposure Phase 2 (Figure 4-3). There was a significant effect of the interaction between experimental group and phase on mean degree (LMM group*phase, $F=7.3343$, $p=0.001$). In control colonies there was a significant increase in mean degree over time (Tukey post-hoc contrasts: Phase 3 - Phase1, $p<0.001$; Phase 3 – Phase 2, $p=0.041$). This suggests that the mean degree of proximity networks increases with network size under natural conditions. Treatment colonies however showed a significant decrease in mean degree between the baseline Phase 1 and the exposure Phase 2 ($p<0.040$). This decline was followed by a significant increase in mean degree between Phase 2 and the post-exposure Phase 3 ($p<0.001$). However, a direct comparison of mean degree between control and treatment colonies showed no significant difference during Phase 2 ($p=0.346$). Nevertheless, the observed pattern within the treatment group is consistent with an effect of the pesticide treatment on a decrease in the number of interaction partners per hour.

In HTH networks, mean degree was constant over time in control colonies, but there is strong evidence that exposure to pesticides decreased mean degree in treatment colonies. There was a significant effect of the interaction between experimental group and phase on mean degree in HTH networks (LMM group*phase, $F=10.2$, $p<0.001$), suggesting per phase differences in mean degree. In control colonies there were no significant differences in mean degree between Phase 1 and Phase 2 ($p=0.999$), Phase 2 and Phase 3 ($p=0.923$), or Phase 1 and Phase 3 ($p=0.971$; Figure 4-3). This

suggests that there is a limit to the rate of engaging in HTH interactions with new interactions partners over a range of network size, i.e. mean degree in HTH networks is independent of network size. In contrast, there was a significant decrease in the mean degree of treatment colony networks between the baseline Phase 1 and the exposure Phase 2 ($p<0.001$). Additionally, the mean degree of treatment colony networks was significantly lower than control colony networks during the exposure Phase 2 ($p=0.022$). This strong effect of pesticide exposure on the mean degree of HTH networks was also completely reversed during the post-exposure phase. There was no significant difference between control and treatment colony networks during the post-exposure Phase 3 ($p=0.992$), or between treatment Phase 1 and treatment Phase 3 ($p=0.827$). These results provide strong evidence that exposure to pesticides reduced the number of unique HTH interaction partners per bee per hour, but that colony interaction networks recovered after exposure and mean degree returned to its pre-exposure level.

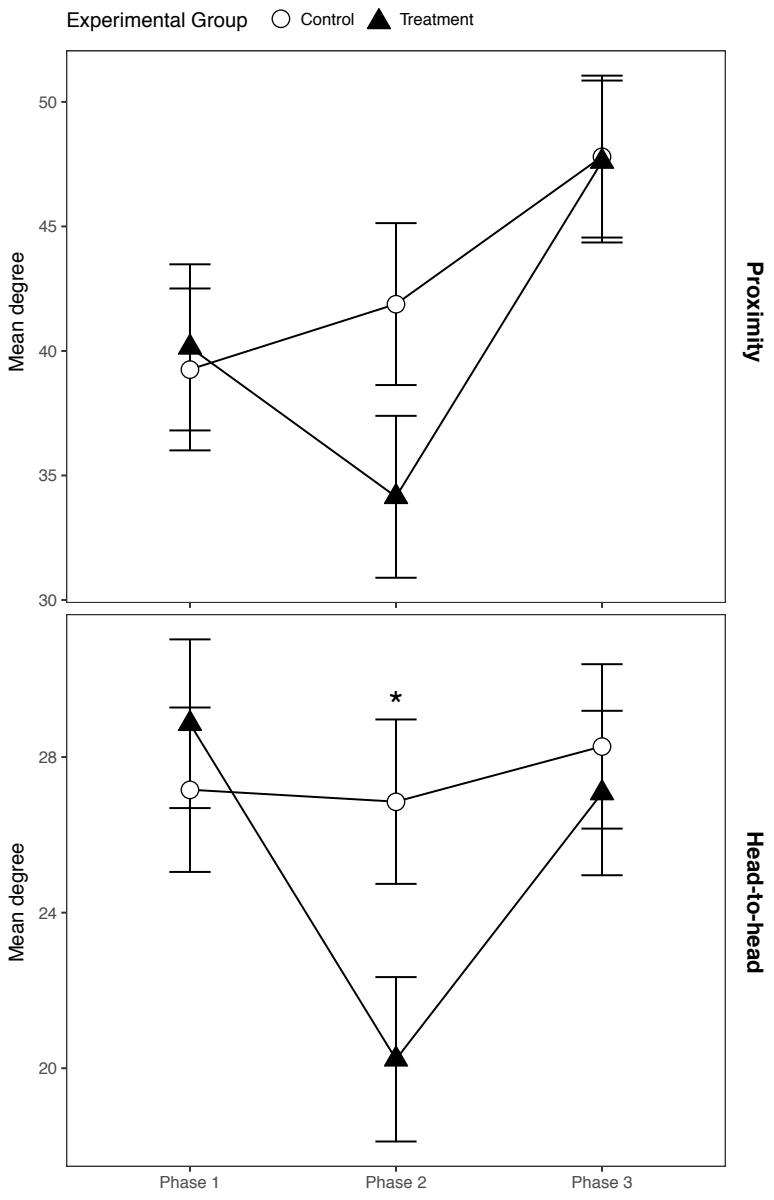

Figure 4-3. Mean degree decreases during pesticide exposure and recovers post-exposure. Points show mean values per phase of colony network mean degree, estimated from a linear mixed model (see text). Error bars show 95% confidence intervals. Top panel shows the mean degree of proximity networks, while the bottom panel shows the mean degree of head-to-head networks. Asterisks denote statistical significance between control (open circles) and treatment (closed triangles) within phases.

4.3.3 Interaction Rate

Within networks of proximity interaction there was no significant effect of either experimental group (LMM group, F=0.049, p=0.83) or phase (LMM phase, F=2.174, p=0.156) on mean strength (Figure 4-4). This suggests that interaction rate remains stable over time as network size increases and was not affected by pesticide exposure during.

The mean strength of HTH networks was also stable over time in control colonies group but mean strength appeared to decline during pesticide exposure and recover post-exposure in treatment colonies (Figure 4-4). Overall, there was a significant effect of the interaction between experimental group and phase on mean strength (LMM group*phase, F= 3.787, p=0.026), suggesting the effect of experimental group varied per phase. In the control group there were no significant differences between any of the experimental phases: Phase 1 and Phase 2 (Tukey post-hoc contrasts, p=0.915), Phase 2 and Phase 3 (p=0.836), Phase 1 and Phase 3 (p=0.739). Within the treatment group however mean degree decreased during the pesticide Phase 2, but seemed to recover fully during Phase 3. Mean strength during the exposure Phase 2 was significantly lower than Phase 1 ($p<0.001$) and Phase 3 (p=0.041). This effect is consistent with, however there were no significant differences in mean strength between the control and treatment group within Phase 1 (p=0.9148), within Phase 2 (p=0.8356) or within Phase 3 (p=0.979). These data suggest that pesticide exposure altered the mean frequency of head-to-head interaction per bee within treated colonies and that this effect was reversible post-exposure.

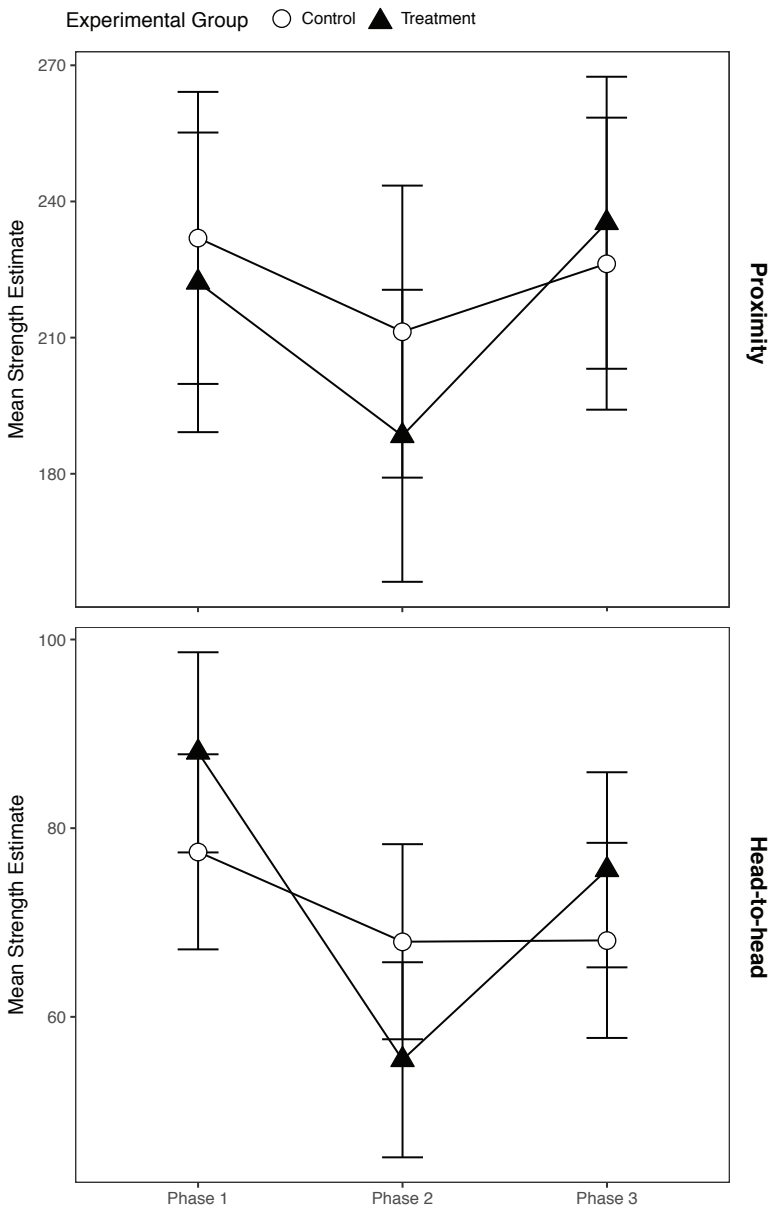

Figure 4-4. Mean strength of head-to-head networks decreased during pesticide exposure and recovered post-exposure. Points show mean values per phase of colony network mean strength, estimated from a linear mixed model (see text). Error bars show 95% confidence intervals. Top panel shows the mean strength of proximity networks, while the bottom panel shows the mean strength of head-to-head networks.

4.3.4 Task Group Mixing Patterns

The task-group mixing patterns were significantly different from the null model in almost every single proximity network, however there is little evidence of consistent deviations from random mixing (Figure 4-5). According to the variance estimates from the jackknife procedure, all calculated values of the inter-category mixing coefficient s from the control group were statistically different from zero. Despite the strong confidence in the calculation of each value of s, most values were near zero and values appear to be equally distributed above and below the level of random mixing. The exception to this was the control Colony J, which scored values of s below zero in all cases, which is strong evidence of significantly less mixing between task-groups than expected based on a null model of randomised network edges. However, in the other three control colonies, 19/45 values of the coefficient s were significantly above zero, while 26/45 were significantly below zero. Despite the statistical significance of each individual value of s (with respect to 0), it was not possible to reject the null hypothesis that the values were equally distributed above and below zero (two-tailed exact binomial test of 19 versus 26, p=0.371). When Colony J was included in this test, the proportions of values above and below zero were 19/60 and 41/60, respectively, there were significantly more values below zero than under the null hypothesis of 0.5 (two-tailed exact binomial test of 19 versus 41, p= 0.006). This indicates that foragers and non-foragers in most bumblebee colonies interact randomly with respect to task group, but there is colony variation.

In proximity networks of the treatment group, all recorded values of s were also significantly different from zero, according to the estimated

variance, except for the value of *s* from the network of the treatment Colony M on the pre-exposure Day 4 (2.11×10^{-5}, 95% CI=-3.49×10^{-5}, 4.56×10^{-6}).

There was no evidence that pesticide exposure altered task-group interaction patterns in either proximity networks. Firstly, in proximity networks there was a significant effect of the interaction between experimental group and phase on the coefficient *s* (LMM group*phase, F=3.634, p=0.030), but this effect was attributable to a significant decrease in *s* between Phase 1 and Phase 3 in control colonies (Tukey post-hoc contrast, $p<0.001$). This result suggests that the coefficient *s* significantly decreased over time in control colonies (Figure 4-5). Despite this effect, there was no evidence for an effect of pesticide exposure on the *s* coefficient. There were no significant differences between phases within the treatment group, nor were there any significant differences between the control and treatment groups within each phase according to multiple pairwise post-hoc contrasts. When the outlier Colony J was excluded from the analysis, there was no overall effect of the interaction between group and phase (LMM group*phase, F= 2.127, p=0.126), nor were there any significant post hoc results between any pairwise group and phase comparisons.

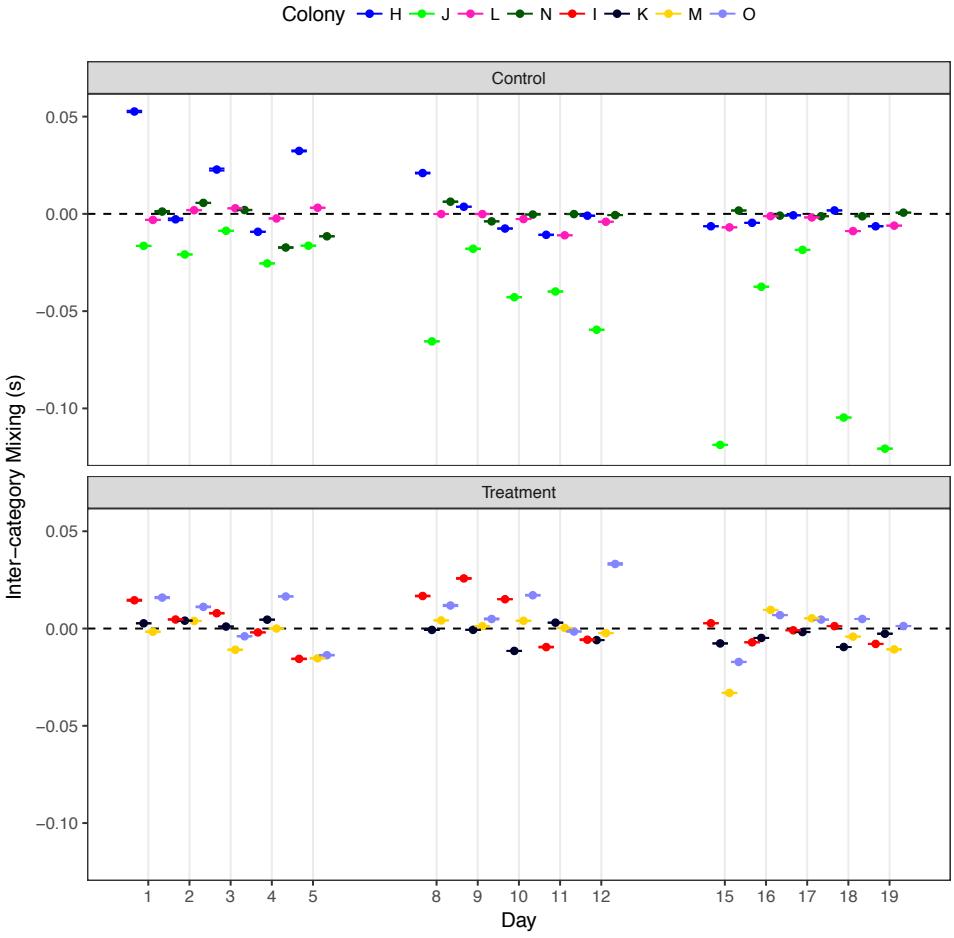

Figure 4-5. Bumblebees interact randomly with respect to forager/non-forager task groups and this pattern is not affected by pesticide exposure. The coefficient of inter-category mixing s is positive when more interactions occur between foragers and non-foragers than expected according to the null model of random interactions with respect to task group, and is negative when there is less inter-category mixing than expected. Points show daily measurements of s per colony and are offset horizontally to avoid overlap. Days shown on the x-axis represent each experimental phase (Phase 1 = day 1-5, Phase 2 = day 8-12, Phase 3 = day 15-19). Error bars show 95% confidence intervals estimated via a jackknife procedure.

4.3.5 Information Flow

4.3.5.1 Curve Fitting & Model Selection

The SI model of information flow generated a total of 119 information flow simulations, resulting in data describing 60 information flow growth curves from the control group, and 59 curves from the treatment group. An example of the curve fitting for candidate functions is shown in Figure 4-6. The mathematical model that achieved the lowest BIC score for the most information flow growth curves was the hyper-Gompertz function (76/119; see Table 4-1). The number of best-fitting hyper-Gompertz curves was evenly spread different between the control (40/76) and treatment group (36/76) (two sample t-test, t=1.284, df=5.028, p-value = 0.2552). This shows that the model is not biased to fit just one experimental group and that it will work well as a general model to describe information flow in control and treatment colonies.

The specific parameterisation of the hyper-Gompertz function used was that described by Tsoularis and Wallace (2002). This function, also known as the 'generalised Gompertz' or the 'generalised ecological growth function', is given by the differential equation,

$$\frac{dN}{dt} = rN\left[ln\left(\frac{K}{N}\right)\right]^{\gamma},$$

where $\gamma>0$ (Turner et al., 1976). In the case where $\gamma = 1$ this equation simplifies to the ordinary Gompertz growth function, which was originally developed to describe human mortality (Gompertz, 1825), but has since been applied to model growth in a wide variety of biological systems (see references in Tjørve and Tjørve, 2017). The parameter r is the model specific growth

constant (see Figure 4-7), and therefore describes the rate of information transmission across the temporal network: increasing values of r produce a steeper the curve and thus a faster rate of information flow. This parameter can be compared between hyper-Gompertz models but may not be directly comparable to the growth constants of other traditional growth models (Tjørve and Tjørve, 2017). Additionally, the parameter γ is only interpretable as an additional 'shape' parameter of the hyper-Gompetz function (Figure 4-8). Values of γ approaching 1 produce more 'S-shaped' curves, while increasing values of γ produces more 'exponential-shaped' curves. The power of these two parameters is that they describe the wide range of shapes seen in the information flow curves and they can be used to compare flow dynamics between networks.

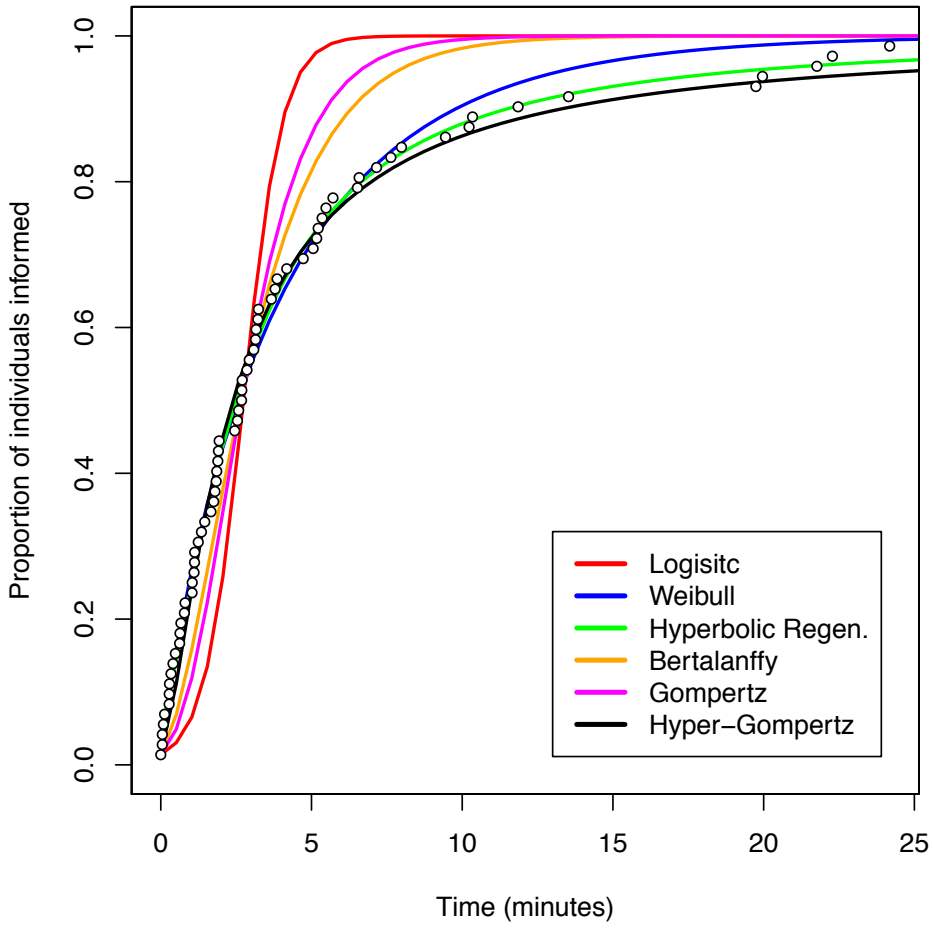

Figure 4-6. Fitted candidate growth functions. The six tested candidate growth function fitted to an illustrative example of colony information flow data (Colony I, day 19).

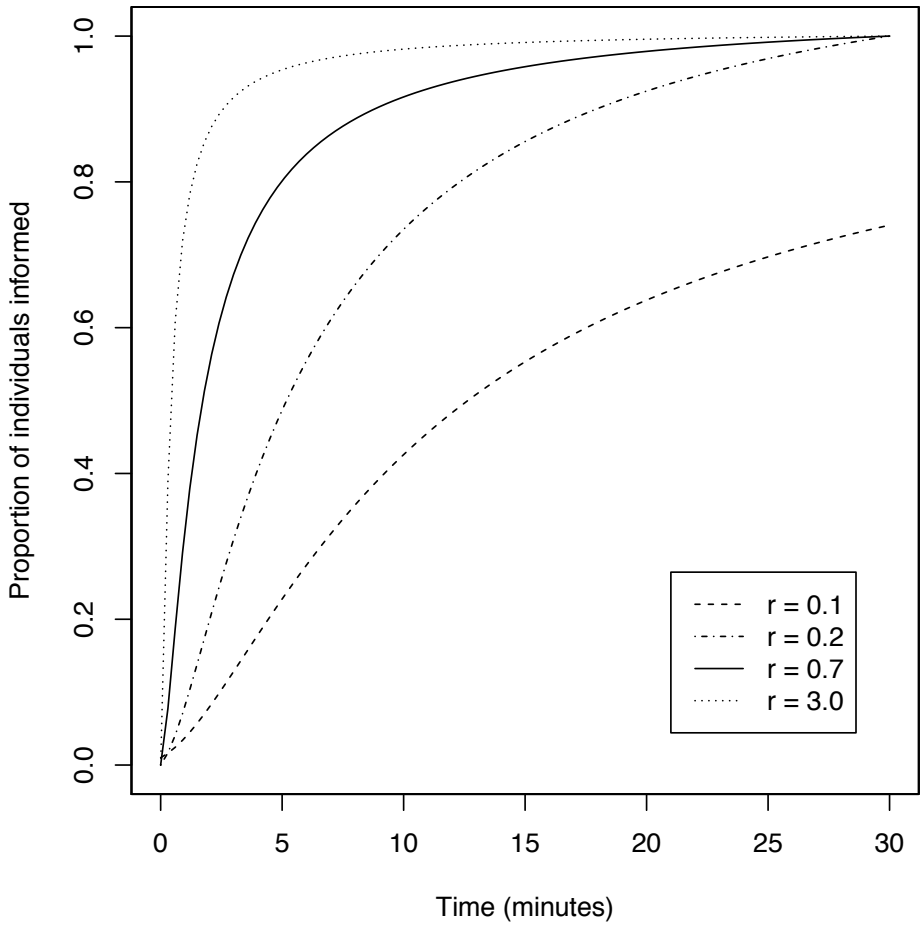

Figure 4-7. Variation in the hyper-Gompertz parameter *r*. Examples of the growth in the proportion of informed individuals as a function of time for different values of the parameter r in the hyper-Gompertz model. In all cases $K = 1$ and $P_0 = 0.01$, which provides a simulation of a single informed forager entering a colony of 100 bees total. The parameter $\gamma = 1.9$ in all curves, which was the mean γ from the empirical information flow curves. The parameter r varies $r = 0.1$, $r = 0.2$, $r = 0.7$, r=3.0. Values of r represent a sample from the range of r parameter estimates from the empirical information flow curves. The x-axis is scaled to show only 30 minutes to show curves more clearly.

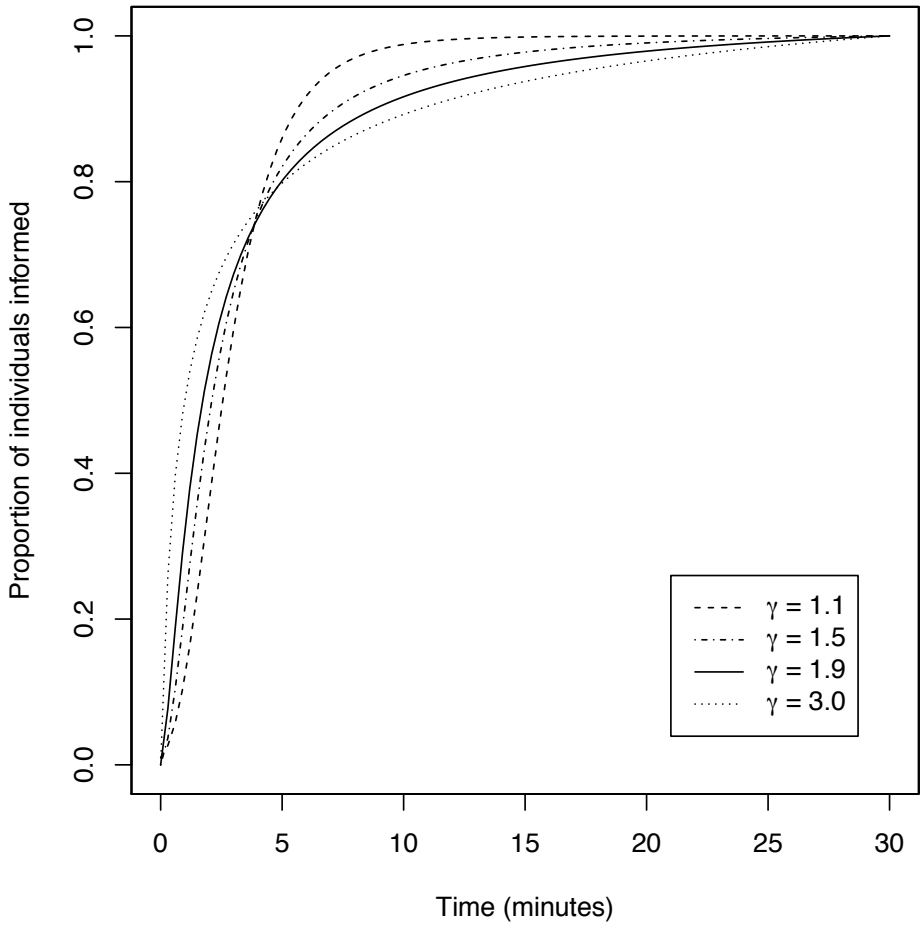

Figure 4-8. Variation in the hyper-Gompertz parameter γ. Examples of the growth in the proportion of informed individuals as a function of time for different values of the parameter γ in the hyper-Gompertz model. In all cases $K = 1$ and $P_0 = 0.01$, which provides a simulation of a single informed forager entering a colony of 100 bees total. The parameter $r = 0.7$ in all curves, which was the mean r from the empirical information flow curves. The parameter γ varies $\gamma = 1.1$, $\gamma = 1.5$, $\gamma = 1.9$, $\gamma = 3.0$. Values of γ represent an approximation of the range of γ parameter estimates from the empirical information flow curves. The x-axis is half of the 1-hour observation period to show the shape of the curves more clearly.

4.3.5.2 Information Flow Model Parameters

Pesticide exposure did not significantly affect the flow dynamics of temporal networks in bumblebee colonies, although there were some changes to the shape of the modelled information flow growth curves (Figure 4-9). Flow properties were quantified via the r and γ parameters of the fitted hyper-Gompertz curves.

For the growth constant r, there was a significant negative effect of phase on the parameter value across all colonies (Figure 4-10; LMM phase, F=6.364, p=0.013). However, there was no effect of the interaction between experimental group and phase which suggests that r was affected equally with respect to phase in both control and treatment groups. The overall reduction in r over time describes a reduction in the rate of the potential upper bound of information flow as the colony size/number of nodes increases. The average number of interactions per bee (mean strength) was not affected by increasing network size, therefore this reduction in r was probably due to the fact that information flow was measured as a proportion of informed individuals and not as the sum of informed individuals.

There was some evidence of an effect of pesticide exposure of the 'shape' parameter γ. There was a significant effect of the interaction between experimental group and phase on γ (Figure 4-10; LMM group*phase, F=3.58, p=0.03), which suggests γ was affected differently over the phases with respect to control and treatment. In the control group, there was no change in γ over time. This was shown by pairwise comparisons between phases within the control group: there was no significant difference between Phase 1 and Phase 2 (Tukey post-hoc contrasts, p=0.914), between Phase 1 and Phase 3 (p=0.585), and between Phase 2 and Phase 3 (p=0.990). Within

treatment colonies however, there was a significant increase in γ between the baseline Phase 1 and the pesticide exposure Phase 2 (Tukey post-hoc contrasts, $p<0.001$). Following the increase during Phase 2, there was no change in γ between Phase 2 and the post-exposure Phase 3 ($p=1.000$). These results within the treatment group suggest that pesticide exposure may have caused an increase in γ during Phase 2, which persisted into Phase 3. Despite the increase within the treatment group during Phase 2, there was no significant difference in γ between control and treatment groups during Phase 1 ($p=0.944$), during Phase 2 ($p=0.486$), or during Phase 3 ($p=0.872$). Additionally, the effect on the 'shape' parameter γ within the treatment group was small (treatment Phase 1, $\gamma=1.665$ 95% confidence interval (CI) [1.510, 1.819]; treatment Phase 2, $\gamma=2.132$, CI [1.981, 2.283]). The effect of this change in γ on the dynamics of $P(t)$ (on the scale of minutes) would be small.

This model is able to quantify the dynamics of the potential for information flow in bumblebee temporal networks and allows useful comparisons to be made, but ultimately, it is somewhat difficult to relate the parameters to the mechanics of bumblebee behaviour. A more detailed model, tailored to incorporate the mechanics of information flow in colonies might be needed to best understand how information flows and differs between colonies.

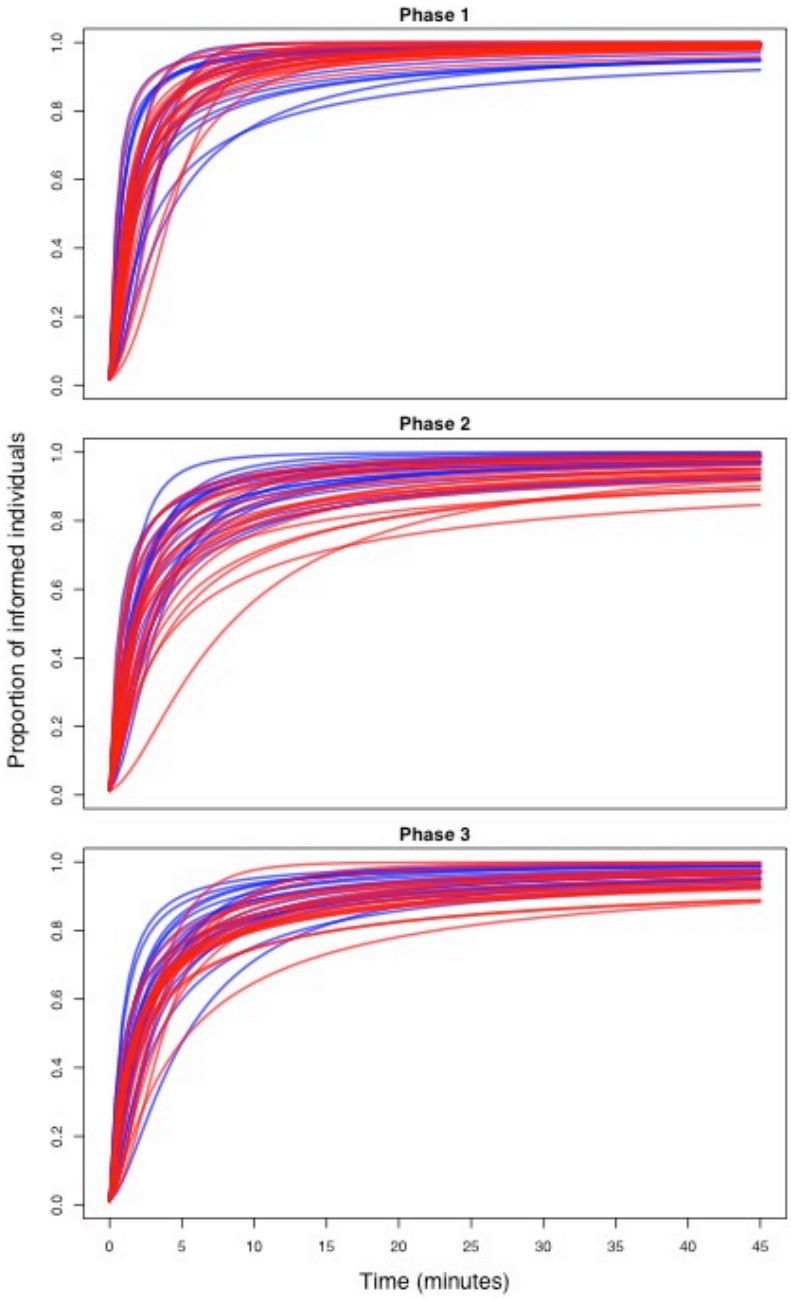

Figure 4-9. No change in information flow curves during pesticide exposure. Information flow curves modelled by the hyper-Gompertz function. Blue curves are from control colonies; red curves are from treatment colonies. The x-axis shown in the range 0-45 minutes for display purposes. Phase 1 = pre-exposure; Phase 2 = pesticide exposure; Phase 3 = post-exposure.

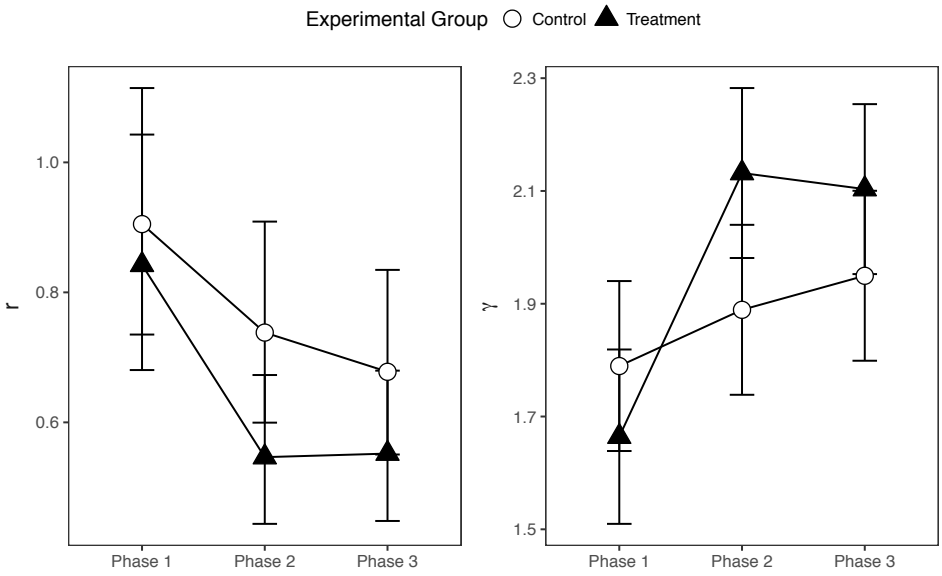

Figure 4-10. Trend of decreasing hyper-Gompterz r parameter and trend increasing γ parameter during pesticide exposure. Means of the two hyper-Gompertz function parameters estimated by linear mixed-effects models (LMM). The estimates of the parameters r and γ were from an LMM the included the interaction between experimental group and phase as the fixed effect, plus colony as a random effect. Error bars show 95% confidence intervals.

4.4 Discussion

Networks of interactions are fundamental to social insect colony self-organisation (Naug, 2015). Recorded social networks in social insects appear to exhibit both flexibility and robustness in the face of disturbances (Jeanson, 2012; Naug, 2009). This study shows that exposure to the common agricultural pesticide imidacloprid has the potential to disrupt social interactions in colonies of the bumblebee *Bombus terrestris*, but that colony interaction networks may show some resilience in terms of stable patterns of task group interactions and a preservation of rapid information flow.

Social mixing, as measured by mean degree, describes the average diversity of interaction partners across the colony, and thus, the extent to which individuals in the colony are mixed. In networks of proximity interactions there was a decrease in this measure of social mixing that was consistent with an effect of the exposure to pesticides. The effect was even stronger in HTH networks, where social mixing was significantly lower during pesticide treatment than in control colonies. Taken together, these results suggest that neonicotinoid exposure decreases the rate at which bumblebees interact with different colony members. This effect is consistent with predictions made based on the results of Chapter 3, namely that during exposure bees moved more slowly in smaller areas of the nest. It seems this effect on movement and space use has constrained the bees to interact within less diverse clusters, which is hypothesised to restrict the flow of information across the colony (Blonder and Dornhaus, 2011). While bumblebees show some spatial fidelity (Crall et al., 2018; Jandt and Dornhaus, 2009), the extent of social mixing is relatively high and interactions across normal colonies are common (van Honk and Hogeweg, 1981). In proximity networks, mean degree increased over time at network size increased in control colonies

(and as shown by the recovery of treatment colonies which matched the increase seen in control colonies).

In contrast, mean degree in HTH networks was stable over increasing network size. This amounts to increase in the average diversity of physical contacts in line with an increase in worker density on one hand, and a stable average of more specific head-to-head contacts over increasing worker density on the other hand. This suggests that HTH interactions are not simply a random subset of proximity interactions, but that this network is describing a different layer of bumblebee social organisation. These differences in the two interaction network types could reflect the relationship between spatial fidelity and dominance in bumblebee colonies. HTH interactions were designed to approximate antennation interactions, which are related to dominance in bumblebee colonies (Sibbald and Plowright, 2014; van Doorn and Heringa, 1986; van Honk and Hogeweg, 1981). These dominance interactions primarily occur between dominant individuals that maintain close spatial proximity to the queen near the nest centre (known as 'elite' workers), while subordinate bees tend to occupy the periphery and do not engage in dominance interactions (Hogeweg and Hesper, 1983; van Honk and Hogeweg, 1981). The stability of HTH interaction partners could represent consistency in the elite group in the nest centre, while the increase in the proximity interaction partners could reflect the fact that more bees are mixing around the nest periphery as worker density increase. Nevertheless, pesticide treatment caused a significant decrease in the number of interaction partners in both contact and HTH networks, which seems to be caused by spatial clustering (Chapter 3).

There was no effect of pesticide exposure on interaction rate (mean strength) in proximity networks. On the contrary, proximity interaction rates

appeared to be stable over both increasing colony size and through the individual locomotor impairments described in Chapter 3. In this study worker populations more than doubled in some cases, but the average interaction rate remained unchanged. This stability in interaction rates suggests that there may be some social mechanism that regulates colony-level contact rates. Such a mechanism would most likely be a simple "behavioural algorithm" at the level of the individual (Sumpter, 2006). For example, individual-based models of group formation show that just two behavioural algorithms are sufficient to generate dynamic aggregations of individuals that behave like fish schools and bird flocks (Couzin et al., 2002). The rules of this particular model can be summarised as: 1) individuals attempt to maintain a minimum distance between themselves and other groups members, and 2) individuals will tend to be attracted towards, and align themselves with other group members [if not avoiding collisions according to rule 1] (Couzin et al., 2002). The result is a relatively stable distance between group members. In a similar way, bumblebees may respond the rate at which they contact other bees by adjusting their locomotor behaviour and maintaining their individual contact rate between some upper and lower bounds. Contact rate regulation has been described in ants (Gordon et al., 1993) and may represent a colony-level property that is resilient to large changes in individual density and, in this case, individual impairments to movement speed (Chapter 3).

Interaction rate in HTH networks showed evidence of a decrease during pesticide exposure, followed by an increase during recovery, but was not significantly different from the control groups during the exposure phase. Qualitatively, the trends in interaction rate between phases appear similar in both network types. This similarity would against suggest that interaction

rates are stable, even if HTH interactions occur more often within an elite group.

Contact interactions between bumblebee foragers and their nest mates have been shown to increase forager recruitment (Dornhaus and Chittka, 2001; Renner and Nieh, 2008). Therefore, the mixing patterns of foragers and non-foragers were quantified before, during and after pesticide exposure to test the resilience of colony-level task group interaction patterns to this disruption. Overall, the results suggest that the observed frequencies of interactions between foragers and non-foragers tend to reflect the frequencies that would be expected based on a model of random mixing between groups. In other words, there was even mixing between task groups in control colonies and there was no observable effect of pesticides on mixing in treatment colonies. This random task-group mixing in control colonies (and pre-treatment colonies) was somewhat expected given that returning foragers are known to run excitedly throughout the nest making many social contacts with many other bees (Dornhaus and Chittka, 2001). However, given the strong negative effect of pesticide exposure on forager movement speed inside the nest (Chapter 3) it is surprising that these task-group interaction patterns show no significant directional deviation from random during pesticide treatment. Although, the effects of pesticide exposure on the spatial centrality of foragers may provide some explanation to these stable mixing patterns during exposure. Active foragers (≥10 foraging bouts over 5 days) showed a clear shift from more peripheral nest occupancy to more central nest occupancy during pesticide exposure (Chapter 3). Similarly to the proposed regulation of interaction rates, foragers could be adjusting their excited run by moving into more central nest areas where the local density is high enough to match their normal interaction rate threshold. Alternatively, foragers could

adjust the time spent inside the nest between foraging bouts in order to match a threshold sum of contact interactions before they leave on another bout. Individual bumblebee foragers have to make frequent optimisation decisions based on thresholds as they fly between variably rewarding flowers (Hodges, 1985; Whitney et al., 2008) and foraging patches (Woodgate et al., 2016). During pesticide treatment, foragers spent significantly longer inside the nest between bouts (Chapter 3), and the interaction patterns with non-foragers were unaffected, thus a social contact threshold could factor in to forager decision-making inside the nest.

Information flow can be considered an emergent property of localised social interactions embedded within a wider colony-level interaction network. Here, the theoretical upper bound of foraging related contact-based information flow was measured by simulating information transmission during pairwise, time-ordered interactions in a temporal network (Blonder et al., 2012). This approach tracked the growth in the proportion of individuals reached by the simulated information and found some non-significant trends of disruptions to flow during pesticide exposure. Flow was quantified by the r and the γ parameters from the hyper-Gompertz growth model (Tsoularis, 2001). There appeared to be decreases in modelled values of the r 'rate' parameter during exposure, which could correspond to a decrease in the rate of interactions that transmit information (Figure 4-7). There also appeared to be an increase in the γ 'shape' parameter (Figure 4-8), which represents a shift in the shape of the information flow curve from more of an 'S-shape' to an 'asymptotic exponential shape'. The asymptotic exponential shape has a slightly more rapid initial growth, followed by an earlier decline in growth (see Figure 4-8). These trends could be explained by some of behaviour observed during exposure in Chapter 3 and the results described above.

Firstly, the rapid initial growth suggested by an increase in γ could be a result of foragers entering the nest with information and moving quickly into areas of high worker density. The interactions as a result of this behaviour could lead to high rates of local information flow (Blonder and Dornhaus, 2011). A decrease in r would follow as a consequence of clustered individuals because the presence of spatially isolated cliques is known to reduce the global flow of information in social insect systems (Blonder and Dornhaus, 2011; Mersch et al., 2013). Ultimately however, the observed changes in these parameters during exposure were small and non-significant, suggesting that colony information flow could be resilient to significant individual impairments in locomotor behaviour. This resilience seems to be a product of simple behavioural algorithms at the individual level that maintain stable interaction rates despite changes in colony demographics and exposure to neonicotinoids.

In summary, this study demonstrates the value in taking a network approach to understanding complex systems. The behavioural impairments of individuals shown in Chapter 3 indicated that colony function could be as severely affected. However, we find that predicting the complex behaviour of the colony is not possible given the sum of the behaviour of the individuals. Strengthening this concept in pesticide risk assessment research is vital to understanding why colonies respond the way they do when their workers are chronically impaired. This study has described the resilience of the potential for information flow in bumblebee colonies during pesticide exposure, but this does not mean neonicotinoids are safe. What this does mean is that bumblebee colonies in large-scale field studies that have suffered reduced colony growth and queen production (Rundlöf et al., 2015; Whitehorn et al., 2012; Woodcock et al., 2017), must have been composed of severely

behaviourally impaired individuals that pushed these colonies past the limits of social resilience.

Chapter 5

Effects of Neonicotinoids on Bumblebee Dominance Hierarchies

5.1 Introduction

Dominance hierarchies in animal groups are emergent social structures of the interactions between individuals faced with reproductive conflict. When reproductive conflict has not been resolved in a group, interactions may be aggressive, but the resulting hierarchy formation helps to avoid future conflict (Aureli et al., 2000). Dominance hierarchies are predominately linear, with a single alpha individual often occupying the top ranked position (De Vries et al., 2006; McDonald and Shizuka, 2013). The alpha position confers many important benefits on the individual including improved access to resources and mates (Herrera and Macdonald, 1993; Muehlenbein and Watts, 2010) and, in some cases, the complete monopolisation of reproduction (Clarke and Faulkes, 1997; Strassmann, 1981). Maintaining the alpha position, however, can be a demanding role and has been associated with costs to the individual in terms of elevated stress (Gesquiere et al., 2011; Muehlenbein and Watts, 2010). Benefits to the alpha tend to come at a cost to subordinates, who are excluded from high-dominance privileges such as access to food, and can also experience high levels of stress and risk of injury due to aggression directed

down the hierarchy (Creel, 2001; Nakano, 1995). Receiving social aggression in humans increases individual disease risk, which can contribute to cause of death (Felitti et al., 1998). Ultimately, behavioural dominance interactions affect group-level social organisation, which can result in differential costs and benefits to group members and affect survival and fitness.

Reproductive skew is one of the hallmarks of hymenopteran insect societies, in which one or a few females maintain reproductive dominance while the rest of the female nest mates remain sterile (Vehrencamp, 1983). In most highly eusocial species the queen position, at the top of the reproductive dominance hierarchy, is uncontested because female workers gain more indirect fitness benefits from helping to rear a female-biased sibling brood than their own sons (Hamilton, 1964). However, in species with more simple societies (where all individuals are capable of mating and laying eggs) this reproductive conflict between queen and workers is not resolved and can result in competition over reproductive rights (Bourke, 1988a; Ratnieks et al., 2006). Workers compete for the chance to reproduce by directing aggression toward the other workers and/or the queen in colonies of some species of wasps (Loope, 2015; Strassmann et al., 2003), ants (Bourke, 1988b; Grainger et al., 2014; Monnin, 1999) and bees (van Doorn and Heringa, 1986; van Honk and Hogeweg, 1981). These competitions result in self-organising, linear dominance hierarchies that help to avoid outright aggression and establish the reproductive dominance of the alpha individual (Shizuka and Mcdonald, 2015).

Given the importance of dominance hierarchies in establishing and maintaining social structure, function, cohesion, and ultimately individual fitness, any factors that disrupt the hierarchy (and specifically the mechanisms by which the hierarchy is formed and maintained) can result in

significant costs to individual fitness. Such factors include intrinsic factors like demographic changes [e.g. through the loss of the queen or other high ranked individuals (Chandrashekara and Gadagkar, 1992)] or extrinsic factors like chemicals in the environment that might disrupt the machinery underpinning cognition and behaviour [e.g. the impact of pesticides on the behaviour of social insects (Pisa et al., 2014)]. The costs and benefits of disruption to fitness could also be affected by an individual's hierarchy position. For example, exposure to a polluting pharmaceutical estrogen at environmentally relevant concentrations increased the reproductive success of dominant female zebrafish (relative to subordinates), while the reproductive skew of male fish decreased (Coe et al., 2008). The formation of dominance hierarchies is well studied, but less is known about their resilience to disturbance, which may be important in predicting the success of social groups or populations faced with anthropogenic change.

5.1.1 Dominance Hierarchies in Bumblebees

In bumblebee (*Bombus* spp.) colonies, female workers cannot mate but they do possess functional ovaries and will attempt to lay male eggs at a late stage of the annual colony lifecycle (Benton, 2006). The period of worker egg-laying is termed the competition phase and is marked by overt aggression between workers and the queen, oophagy by workers and the queen, and may also result in matricide (Duchateau and Velthuis, 1988; van Doorn and Heringa, 1986; van Honk and Hogeweg, 1981). The initiation of the competition phase is a complex emergent property of multiple social cues including the eclosion of gynes, the queen's switch to male production and a decrease in queen inhibition of worker reproduction (Alaux et al., 2004; Bloch, 1999). Worker age and dominance status correlate with egg-laying (van Doorn, 1987; van

Doorn and Heringa, 1986). Dominance status within *Bombus terrestris* colonies is based on the outcomes of agonistic antennation interactions between individuals that occur throughout the colony life cycle (van Honk and Hogeweg, 1981).

The bumblebee colony dominance hierarchy can be replicated in the laboratory within small queenless groups of workers, called microcolonies. Away from the reproductive inhibition of the queen, workers will begin to engage in aggressive contests with one another as they fight for reproductive dominance. Aggressive interactions in bumblebee microcolonies follow attack-retreat contests where one individual initiates aggression ('winner') and the other individual simply retreats ('loser') (Sibbald and Plowright, 2014; van Honk and Hogeweg, 1981). The outcomes of these contests produce a linear hierarchy through "winner-loser" effects (Chase, 1982), where bees that initiate interactions are more likely to 'win' in the future. A single behaviourally dominant bee tends to initiate the majority of interactions and develop her ovaries while suppressing reproduction by her subordinate sisters (Amsalem et al., 2013). This system can also be replicated *in silico*, which has strengthened the view that the behavioural and reproductive hierarchy is an emergent self-organising phenomenon based on the outcomes of local interactions. Models of initially identical, randomly interacting bumblebees affected by "winner-loser" effects have demonstrated that a linear hierarchy can emerge without any external influence (Amsalem et al., 2013; Hogeweg and Hesper, 1983). Thus, it appears that interactions among bumblebee workers make up the building blocks of bumblebee microcolony dominance structures and colony-level social organisation.

5.1.2 The Effects of Pesticides on Reproduction and Dominance in Bumblebees

The reproductive success of bumblebee colonies hinges on sufficient growth in the worker population to help provision brood for the next generation (Benton, 2006). Reported declines of bumblebee populations across the industrialised world would suggest that this complex process is being disrupted (Goulson et al., 2015). Several field studies have suggested exposure to neonicotinoid pesticides could be causing declines by reducing bumblebee (*Bombus terrestris*) colony growth and queen production (Goulson, 2015; Rundlöf et al., 2015; Whitehorn et al., 2012; Woodcock et al., 2017). Similar effects have been reported in laboratory studies, which can, in some cases, provide a more precise view of neonicotinoid effects. Chronic (14 day) exposure to dietary imidacloprid in nectar produced a dose-dependent reduction in the number of brood produced by small, standardised queenright bumblebee colonies (Laycock and Cresswell, 2013) and by small queenless microcolonies (Laycock et al., 2012). Brood production in microcolonies was also reduced by exposure to imidacloprid in nectar (10 ppb; Mommaerts et al., 2010), and in both nectar and pollen (10 ppb and 6 ppb, respectively; Tasei et al., 2000). These studies show clear risks to bumblebee colonies foraging in neonicotinoid-treated agricultural landscapes, but they do not identify the exact mechanism causing reduced brood production.

There are several possible explanations for reduced brood production during neonicotinoid exposure, but there is still confusion in the literature and the possibility of a breakdown in the social structures that underpin colony growth and reproduction has not been investigated. The prevailing view is that brood production is reduced by neonicotinoid-induced nutrient limitation. Laboratory studies have reported that bumblebees in the

neonicotinoid treatment group consumed less food than the control and that there was a positive relationship between food consumed and brood produced (Laycock and Cresswell, 2013; Laycock et al., 2012). Other studies have linked reduced foraging efficiency with reduced brood production, suggesting nutrient limitation may be the mechanism (Gill et al., 2012). One way by which nutrient limitation could reduce brood production is by affecting ovary development. Growing mature oocytes for egg laying is a costly physiological process that relies on energy from nectar carbohydrates and essential nutrients from pollen (Duchateau and Velthuis, 1989). However, Laycock et al. (2012) found no effect of neonicotinoid exposure on ovary development, except at the highest dose of imidacloprid (125 ppb), at the same time as reduced feeding. These findings suggest reduced ovary development is not the primary factor causing reduced brood production. Nutrient limitation could also affect total brood production by increasing brood development time, but there is no evidence for this effect (Gill et al., 2012; Tasei et al., 2000).

Brood production could also be affected by the disruption of the social processes that lead to egg-laying, i.e. dominance interactions. Neonicotinoid exposure has been shown to affect basic bumblebee locomotor behaviour (Chapter 3; Cresswell et al., 2013), which can reduce social interaction frequency (Chapter 4). Reduced dominance interaction frequency induced by neonicotinoid exposure could disrupt the social cues required to form a reproductive dominance hierarchy. One suggestion of the possibility of social disruption was put forward by Laycock et al. (2012). These authors exposed bumblebee (*Bombus terrestris*) microcolonies to a range of concentrations of dietary imidacloprid and found differences in the ovary development and egg-laying of bees according to the pesticide concentration. At the highest concentration (159 ppb) ovaries were not fully developed and bees did not lay

eggs, while at the lower concentrations (>25.4 ppb) ovaries were developed and bees also laid eggs. The interesting result was that at the intermediate dose (63.5 ppb) bees developed ovaries but did not lay eggs. Laycock et al. (2012) related this result to the finding that isolated individual *B. terrestris* workers also develop ovaries but do not lay eggs on their own (Amsalem et al., 2009), which suggests that individuals require social stimuli to initiate egg-laying. This raises the possibility that imidacloprid could disrupt social interactions to the point of repressing individual oviposition. It is also possible that some other individual-level non-social mechanism could have caused this result (Laycock et al., 2012), but the effects of neonicotinoid exposure on social interactions have not been investigated.

Neonicotinoid effects are often recorded as group- or colony-level averages, but this could mask more nuanced effects on behaviour according to social position. Chapter 3 described differential behavioural effects on bumblebees grouped according to foraging effort. This approach revealed that the behaviour of active foragers (≥10 bouts over 5 days) was most strongly affected by neonicotinoid exposure; moreover, active forager movement speed was the only behavioural trait that did not recover post-exposure. Active foragers may be more susceptible to neonicotinoid toxicity because the high energetic demands of flight divert energy from detoxification processes. This was suggested in a study that found workers from microcolonies that were foraging over a 3m distance suffered high mortality rates during neonicotinoid exposure, while those that did not forage showed no mortality (Mommaerts et al., 2010). Dominance position can also affect responses to toxicity. Manson and Thomson (2009) fed bumblebee microcolonies nectar from the flower *Gelsemium sempervirens*, which contains natural toxic alkaloids, and found reproductively subordinate bees suffered significantly reduced ovary

development. In contrast, dominant bees suffer no significant ill effect. This suggests that dominance is also correlated with resilience. Evidence from honeybees (*Apis mellifera*) suggests this could apply to neonicotinoid resilience as well. Honeybees that reacted more aggressively to intruder attack tended to be more resilient to mortality induced by neonicotinoid (acetamiprid) exposure and more resilient to parasitism by the ectoparasitic mite *Varroa destructor* (Rittschof et al., 2015).

Social experience, aggression and ovary development all affect individual bumblebee behaviour through many complex feedback loops (Amsalem et al., 2015). Additionally, bees are exposed to multiple stressors in agricultural landscapes including nutritionally monotonous crops, novel disease, and pesticides ranging from fungicides to acaricides (Dance et al., 2017; Goulson et al., 2015). These stressors have been found to have synergistic effects on colonies (Alaux et al., 2010; Klein et al., 2017; Piiroinen and Goulson, 2016). However the interaction between the stress of social dominance and neonicotinoid exposure in bumblebees has not been tested. This study will explore the potential for dominance status to affect individual susceptibility to neonicotinoid exposure. Understanding these relationships between sociality, behaviour and physiology could also help to clarify the unresolved relationship between neonicotinoid exposure, nutrient-limitation and ovary development by tracking the social interactions during hierarchy formation. This approach to neonicotinoid exposure research, of tracking the responses of social processes such as hierarchy formation at the individual- and group-level should help us to better predict how colonies will respond to exposure in the field (Sponsler and Johnson, 2017).

5.2 Aims and Hypotheses

This chapter tests the overarching hypothesis that the neonicotinoid, imidacloprid, disrupts the dominance hierarchies that are formed by workers in the absence of a queen in bumblebee colonies. This over-arching hypothesis is tested through two aims.

5.2.1 Aim 1: Determine the Effects of Pesticides on Ovary Development

The effects of neonicotinoid pesticides on ovary development will be determined by testing three hypotheses. The first hypothesis will repeat previous work to confirm that imidacloprid exposure (10 ppb) does not have a detectable effect on mean ovary development in bumblebee microcolonies (Hypothesis 1a), as shown by (Laycock and Cresswell, 2013; Laycock et al., 2012). Since the mean ovary development has only ever been considered in previous work, the next two hypotheses test the novel idea that pesticides will affect ovary development differentially depending on the hierarchy position of the individual. Hypothesis 1b states that the ovary development of bees lower in the reproductive dominance hierarchy will be more strongly affected given the combined stress of receiving aggressive interactions and the detoxification of the pesticide. Food consumption was also recorded to test the hypothesis that neonicotinoid-induced nutrient limitation is affecting ovary development (Hypothesis 1c).

5.2.2 Aim 2: Determine the Effects of Pesticides on Agonistic Interactions

Previous work shows that imidacloprid (10 ppb) reduces movement speed (Chapter 3) and social interactions (Chapter 4) in queenright bumblebee

colonies. Following this, agonistic interactions are predicted to occur less frequently in imidacloprid-exposed bumblebee microcolonies than in unexposed colonies (Hypothesis 2a). Subsequently, if agonistic interaction frequency is affected, this could increase the time taken to establish the hierarchy. This leads to Hypothesis 2b, which states that neonicotinoid exposure, by disrupting interactions, will delay the establishment of the hierarchy. Two additional hypotheses will test the idea that an individual's hierarchy position will modulate the behavioural effects of pesticide exposure. Hypothesis 2c states that the dominance interactions of bees lower in the *behavioural* dominance hierarchy will be more strongly affected. Hypothesis 2d states that that the dominance interactions of bees lower in the *reproductive* dominance hierarchy will be more strongly affected. In other words we might expect a steeper hierarchy.

These hypotheses are tested using the key pollinator, *B. terrestris,* but the general predictions could be equally relevant to other species that rely on dominance hierarchies in their social structure and function.

Figure 5-1. Still image taken from one of the videos of a control microcolony on day 2.

5.3 Methods

5.3.1 Microcolony Set-up

Microcolonies (N=27) of *B. terrestris* were established from six laboratory-reared colonies (BioBest N.V., Belgium). Each microcolony was composed of five callow workers (less than 24 hours old) selected from a pool of the six parent colonies. Parent colonies were checked daily for new callow workers. Callow workers, identifiable by their light colouration were used instead of mature adults in order to control for age and social experience: callows are considered to behave as a 'clean slate' (Hogeweg and Hesper, 1983). Another benefit from pooling callow workers from multiple parent colonies is that it reduces genetic bias (Amsalem and Hefetz, 2011). Callow workers within each microcolony were individually tagged with unique visual markers that could be identified by eye. Tag designs were generated by a bespoke 16-bit version of the BEEtag marking system (Crall et al., 2015; see Chapter 2), but were used here for manual observation. Tags were printed on paper squares (3×3mm) and stuck onto the dorsal thorax of each bee with a drop of Loctite® Super Glue Gel.

Microcolonies were housed in ventilated transparent acrylic nest boxes (180x100x100 mm; for more details, see Chapter 2). The floor of the nest-box was lined with paper to provide a tractable walking surface for the bees and to absorb moisture. Microcolonies were supplied with fresh nectar and pollen directly inside the nest box every day. Nectar (sugar water 50% vol/vol) was supplied in two 1.5 ml Eppendorf tubes, each punctured with a small hole to allow the bees to feed. Pollen was provided as a soft dough made by mixing honeybee pollen with a small amount of nectar. Microcolonies were kept in controlled laboratory conditions (24°C) in the dark (to mimic the darkness of

subterranean bumblebee nests), and on electric heated mats [to increase the temperature of the inside the nest boxes to 30°C, which is normal for queenright bumblebee colonies (Vogt, 1986)]. Microcolonies were maintained in these conditions for 5 days which, according to previous studies(Amsalem and Hefetz, 2011; Amsalem et al., 2013), is the appropriate time for behavioural and reproductive dominance hierarchies to become established.

5.3.2 Experimental Design and Data Collection

To test the effect of neonicotinoid exposure on bumblebee dominance behaviour, each microcolony was randomly assigned to either the pesticide treatment group or the control group. The treatment group received the neonicotinoid pesticide imidacloprid in nectar feeders at a concentration of 10 ppb [considered to be a field-realistic concentration (Blacquière et al., 2012)], while the control group received uncontaminated nectar. Treated nectar solution was prepared in the same way as in Chapter 3. Nectar and pollen consumption were measured daily on an electric balance to the nearest 0.001 g.

5.3.2.1 Aim 1: Ovary Development

At the end of the 5-day experiment, bees were killed in a freezer at -20°C and were stored frozen until their ovaries were dissected out. Dissections were carried out under a Leica M165 C stereo microscope in distilled water. The dissected ovaries were photographed with a Leica IC80 HD microscope-mounted camera. Each ovary was composed of four ovarioles, each of which contained several oocytes in various stages of maturity. An index of ovary development was taken as the mean length of the three largest terminal oocytes (at least one from each ovary). This measurement is a commonly used

index of ovary development in bumblebees (Amsalem et al., 2013; Baron et al., 2017a; Bloch and Hefetz, 1999; Padilla et al., 2016). Measurements were taken from the photographs using the measuring tool in the image processing software FIJI (Schindelin et al., 2012) with reference to an appropriate scale for each image. Additionally, bees were ranked within each microcolony in terms of their ovary development, from the largest (rank 1), to the smallest (rank 5) (Amsalem et al., 2013). This ranking will be referred to as the *reproductive dominance hierarchy* as it represents the order of bees most likely to become the first egg-layer.

5.3.2.2 Aim 2: Agonistic Interactions

Behavioural observations were conducted by recording 30 minutes of video from each microcolony every day for five days (150 minutes total). The frequency of agonistic interactions in bumblebee microcolonies has been shown to peak 3 - 4 days after microcolony establishment (Amsalem and Hefetz, 2010). Therefore, five days of behavioural observation will capture the interactions most important in establishing a dominance hierarchy. Video-recordings of the inside of the nest were made with an ultra-high resolution (2160x3840 pixels) SONY FDR AX-100 camcorder at 25 frames per second. The camera was positioned directly above the nest box, looking down through the glass lid into the colony. Four panels of red LEDs (Kingbright L-7104SRC-D, 640nm) were arranged around the outside of the nest to illuminate the nest contents during video recording (Figure 5-1. Still image taken from one of the videos of a control microcolony on day 2.). Bumblebees cannot see red light (Peitsch et al., 1992); therefore, their behaviour was not disturbed during video recording.

Agonistic interactions related to dominance hierarchies in bumblebee groups were scored manually from microcolony video recordings. The interactions included in the analyses are described in Table 5-1. The interactions were all directional, worker-worker interactions that have been recorded previously in microcolonies (Sibbald and Plowright, 2013; Sibbald and Plowright, 2014) and queenright colonies (Duchateau, 1989; Hogeweg and Hesper, 1983; van Honk and Hogeweg, 1981). The interactions are described as 'agonistic' (as opposed to 'aggressive') because this is a general term for competitive interactions that includes threats and displays (e.g. darting, see Table 5-1). A pair of bees engaged in an interaction is referred to as a 'dyad'. In directed interactions, the 'actor' refers to the bee that initiated the interaction and the 'recipient' was the bee that either retreated from the interaction or was the individual towards whom the interaction was directed. Each interaction was considered a contest and actors were considered to have 'won' the interaction (which is interpreted as a relative increase in their dominant position), while recipients were considered to have 'lost' the interaction (Chase, 1982). Any interaction that involved three or more bees was ignored because the direction of the interaction could not be reliably determined. Within any dyad, the actor could sometimes direct more than one type of contact-based agonistic interaction (all except darting) toward the recipient in quick succession. Successive contact-based interactions followed a specific sequence of escalating aggression (antennation −> butting −> biting −> grappling). In cases where the dyad did not break physical contact, the final stage of the interaction escalation was recorded (e.g. if an actor directed a butt followed by a bite while the dyad was still in contact, the bite was recorded). If a dyad broke physical contact between successive interactions, the interactions were recorded separately. The number of times each

individual was the actor across all interactions was used as an individual index of behavioural dominance (Amsalem and Hefetz, 2010).

An observer (F. Cullen) scored all interactions blind to the treatment group of each microcolony. A second observer (S. Duckerin), also blind to treatment, verified the interpretation of each interaction scored by the primary observer. Verification involved replaying the video of the interaction and agreeing on the interaction type to a level of certainty that would ensure repeatability of the results given the written descriptions in Table 5-1. Directed agonistic interactions recorded during microcolony hierarchy establishment. Any interactions with uncertain directionality or categorisation were not included.

The ranked index of behavioural dominance for individuals within each microcolony was used as a *behavioural dominance hierarchy*. Unlike the reproductive dominance ranks, it was not possible to rank the bees in each microcolony on a 5-tier hierarchy because of the high prevalence of ties in the index of behavioural dominance. When bees are tied in their indices, their respective ranks cannot be resolved. Overall, there were 82 bees tied with 0 interactions, 13 bees tied with 1 interaction, and 15 bees tied with 2 interactions (out of a total of 135 bees). The number of microcolonies with sufficient differentiation in the indices of behavioural dominance to define hierarchies of different tiers were: five-tier hierarchy N=4, four-tier hierarchy N=8, three-tier hierarchy N=15, two-tier hierarchy N=24. The remaining three colonies contained zero interactions each. Thus, for the majority of microcolonies it was possible to rank bees according to two hierarchical positions, i.e. one individual was ranked in the alpha position, while the remainder were simply classed as a single behaviourally subordinate group. A two-tier system was also appropriate in this case because a single individual

tends to initiate the majority of interactions in bumblebee microcolonies (Amsalem et al., 2013)

Table 5-1. Directed agonistic interactions recorded during microcolony hierarchy establishment.

Interaction	Description	Reference(s)
Antennation	A pair of bees touch antennae, pause for a moment, then one bee ends the interaction and retreats away from the other. The bee that initiates the interaction (actor) must be different to the bee that retreats from the interaction (recipient). This distinction excludes cases where an active individual touches antennae with a stationary, unresponsive individual.	van Honk and Hogeweg (1981) Sibbald and Plowright (2014)
Darting	One bee (actor) accelerates suddenly in the direction of another bee (recipient) but the actor does not make contact with the recipient.	Duchateau (1989) Sibbald and Plowright (2014)
Butting	One bee (actor) accelerates suddenly in the direction of another bee (recipient) and the actor makes contact with its head against any part of the recipient's body.	Duchateau (1989) Sibbald and Plowright (2014)
Biting	One bee (actor) uses its mandible to grab any part of another bee's body (recipient). Occasionally the actor will pull on the body part of the recipient.	N/A
Grappling	One bee (actor) mounts another bee (recipient) and grabs the recipient with its legs, resulting in both bees tumbling in a somersault motion.	Sibbald and Plowright (2014)

5.3.3 Statistical Analyses

Statistical analyses were performed in R version 3.3.0 (R Core Team, 2016). The differences in the consumption of nectar and pollen between experimental groups were tested with paired t-tests. The effects of rank and experimental group on mean terminal oocyte length (continuous and normally distributed response variable) were analysed using linear models with the R function 'lm'. The sum of agonistic interactions and the index of behavioural dominance (both overdispersed and zero-inflated count response variables) were analysed using generalized linear models (GLM) with a negative binomial error distribution. Microcolony ID was included as a random factor in the generalized linear mixed model (GLMM) used to analyse the daily number of interactions to account for the non-independence of repeated measures on each microcolony. Microcolony ID was also included as a random effect when it explained a significant proportion of residual variance. A likelihood ratio test was used to confirm that each negative binomial model provided a better fit than an equivalent model with a Poisson distribution. Generalised linear models were constructed in the R package 'MASS' (Venables and Ripley, 2002).

5.4 Results

A total of 27 microcolonies were established during the experiment, 13 were in the control group and 14 were in the treatment group. Individual bee mortality occurred in some microcolonies before the end of the 5-day experiment. Four microcolonies in the control group had one bee die in each and three microcolonies in the treatment group that had one bee die in each during the experiment. There was no evidence for an effect of treatment on

the number of microcolonies with recorded mortality (Fisher's exact test, p=1).

5.4.1 Aim 1: Effects of Pesticide Exposure on Ovary Development

Ovarian development (mean 3 largest terminal oocytes) was measured for 60 bees across 13 control microcolonies, plus 66 bees across 14 treatment colonies. It was not possible to measure the ovaries of individuals that died during the experiment because the reproductive tissues degraded rapidly after death due to the heated nest boxes. Any microcolonies with missing ovary measurements were excluded from the analysis regarding ovary ranks because the ranks may not match the ranks in microcolonies with no mortality. This left 40 bees from 8 microcolonies in the control group, and 55 bees from 11 microcolonies in the treatment group. The mean length of the terminal oocyte was used as an index of ovary development and ranged from 0.310 – 1.125 mm in the control group and from 0.207 – 1.122 mm in the treatment group.

Ovary development was differentiated within microcolonies and formed linear reproductive dominance hierarchies (Figure 5-2. Linear reproductive dominance hierarchy is not affected by pesticide exposure.). In the control group, the steepness (the absolute slope of ranked ovary development) was 0.106 (R^2=0.560) and this relationship was significant (LM $F_{1,38}$=50.59, p <0.001). This level of development and linear relationship agrees with previous descriptions of ovary development in 5-day old microcolonies of 10 bumblebees (*Bombus terrestris*, Amsalem et al., 2013). The relationship between rank and ovary development in the treatment group was also significant (LM $F_{1,53}$=54.44, p<0.001) and the steepness was 0.096 (R^2=0.383). There was no evidence for an effect of pesticide exposure on mean ovary development in treatment microcolonies (LM $F_{1,92}$=0.013,

p=0.910), which confirms the previous findings of Laycock et al. (2012) and provides evidence to support Hypothesis 1a. Finally, there was no effect of the interaction between experimental group and rank on mean terminal oocyte length ($F_{1,91}$=0.012, p=0.912); this suggests there was no evidence for a differential effect on ovary development with respect to reproductive dominance rank (Hypothesis 1b). This analysis was repeated to include microcolonies with missing ovary measurements and showed the same trends, but with weaker correlations (i.e. smaller R^2, see Appendix 1)

The consumption of untreated pollen was significantly affected by exposure to pesticide-treated nectar (Figure 5-3). Total pollen consumption in treated microcolonies (N=14) was significantly lower than control microcolonies (N=13) (two-sample t-test; t=2.425, d.f.=19.761, p=0.025).

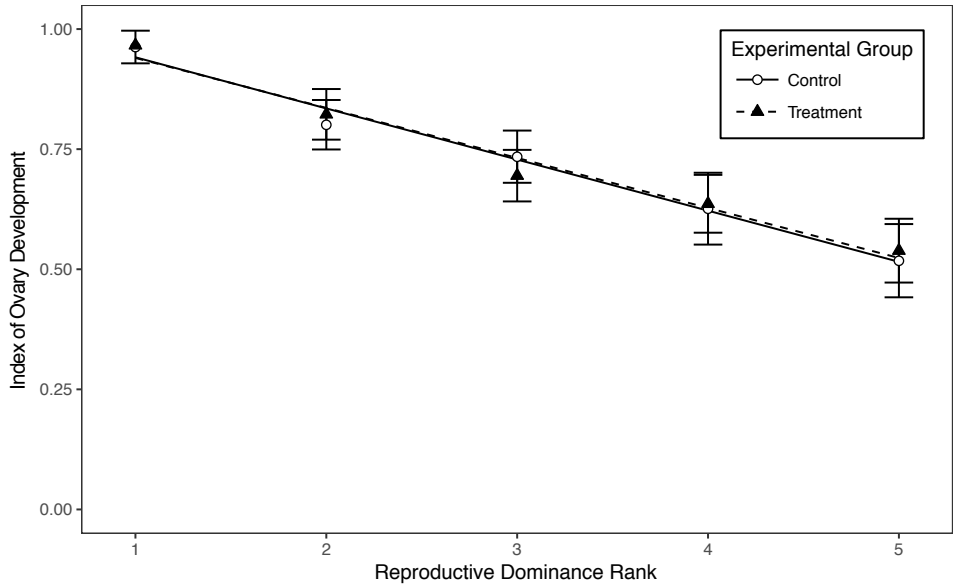

Figure 5-2. Linear reproductive dominance hierarchy is not affected by pesticide exposure. Bees in each microcolony were assigned a rank according to their index of ovary development [mean terminal oocyte length (mm)] from largest (rank 1), to smallest (rank 5). Mean ovary development is shown for each rank in control microcolonies (open circles), and treatment microcolonies (closed triangles). Error bars show ± standard error. Lines show linear regression results; solid line = control, dashed line = treatment.

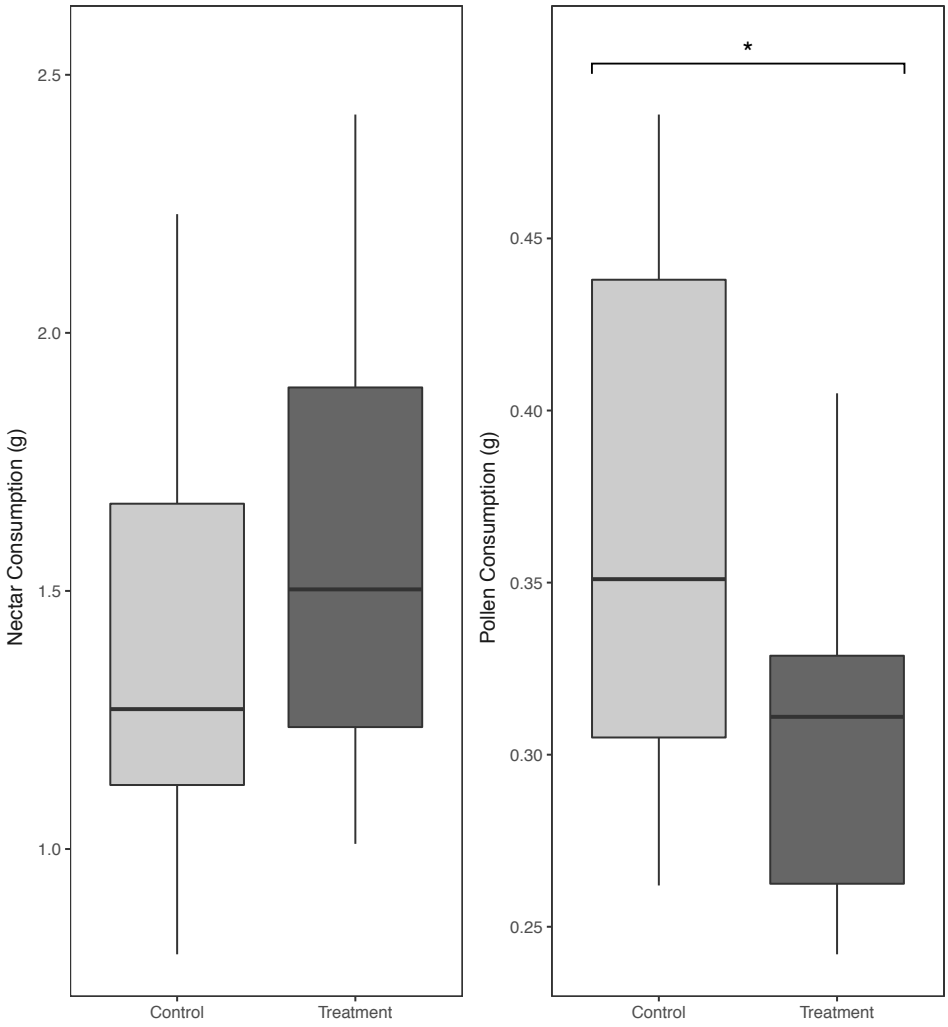

Figure 5-3. Consumption of untreated pollen significantly decreased by exposure to pesticide treated nectar. Asterisk denotes statistical significance at $p<0.05$.

5.4.2 Aim 2: Effects of Pesticide Exposure on Agonistic Interactions

Agonistic interactions were recorded in all microcolonies except three treatment microcolonies that did not display any directed interactions during the observation time. Across all five days of the experiment (150 minutes observation) there were 20.46±4.19 (mean±SE) interactions per microcolony in the control group and 9.21±2.13 interactions per microcolony in treatment group (including microcolonies with zero interactions). This difference in mean number of interactions was significant (GLM χ^2=4.0495, d.f.=1, p=0.044; N=27), suggesting pesticide treatment significantly reduced the number of agonistic interactions in treatment microcolonies (Hypothesis 2a).

In both control and treatment microcolonies the number of interactions per day increased from the beginning of the experiment, peaked on the fourth day, and decreased slightly on the final day (Figure 5-4). Pesticide exposure appeared to reduce the number of interactions on Day 3 and Day 4 relative to the control group. However, the trend was not significant: there was no detected effect of the interaction between experimental group and day on the number of agonistic interactions in bumblebee microcolonies (negative binomial GLMM χ^2=3.268, p=0.514; N=27), suggesting no significant day-to-day differences. Hierarchy establishment is thought to produce a social order that avoids the need for aggression, which would suggest that the time when aggression in a group abates could be considered the time when the hierarchy is established. These data, therefore, suggest that there is no evidence to support the hypothesis that pesticide exposure delayed the establishment of the hierarchy (Hypothesis 2b).

The two-tier behavioural dominance ranks showed that much of the effect of pesticide exposure on reducing interactions was due to the effect on the

behaviourally dominant individuals, as shown in Figure 5-5 (Hypothesis 2c). These results do not include the colonies with zero interactions (N=3), resulting in 13 control colonies and 11 treatment colonies. There was a significant effect of the interaction between behavioural dominance rank and experimental group on the index of behavioural dominance (GLM χ^2=4.654, p=0.031), suggesting differential effects on suppressing interactions according to rank (Hypothesis 2c). A further investigation of the differences in behavioural dominance between control alphas and the treatment alphas was carried out with Tukey post-hoc contrasts. This pairwise test did not show a significant difference in the index of behavioural dominance between the behaviourally dominant bees of control microcolonies and treatment microcolonies (p=0.569). Tukey tests did show however, that dominant bees had a significantly higher index of behavioural dominance than subordinate bees in the control group (p<0.001) and in the treatment group (p<0.001). This confirms that there is also a hierarchy based on dominance behaviour, which determines the level of behavioural changes induced by pesticide exposure. It seems that although behaviourally dominant bees appeared to be more strongly affected by pesticide exposure, this effect may simply represent the fact that behaviourally dominant bees simply have "more to lose" when the number of interactions initiated is the response variable.

Finally, there was a significant relationship between position in the reproductive dominance hierarchy and the index of behavioural dominance (GLM χ^2=19.137, p<0.001), which suggests that bees with relatively more developed ovaries tend to initiate more agonistic interactions (Figure 5-6). This supports previous findings that aggressive bees dominate reproduction in microcolonies (Amsalem and Hefetz, 2011; Amsalem et al., 2013). The effect of pesticide exposure on the index of behavioural dominance appears to be

more severe for reproductively dominant individuals; however there was no significant effect of the interaction between reproductive dominance rank and experimental group (control and treatment) on the index of behavioural dominance (GLM χ^2= 0.079, p=0.778). Therefore, these data suggest there is no evidence for Hypothesis 2d, that position in the reproductive hierarchy could modulate the effect of pesticide exposure on behaviour.

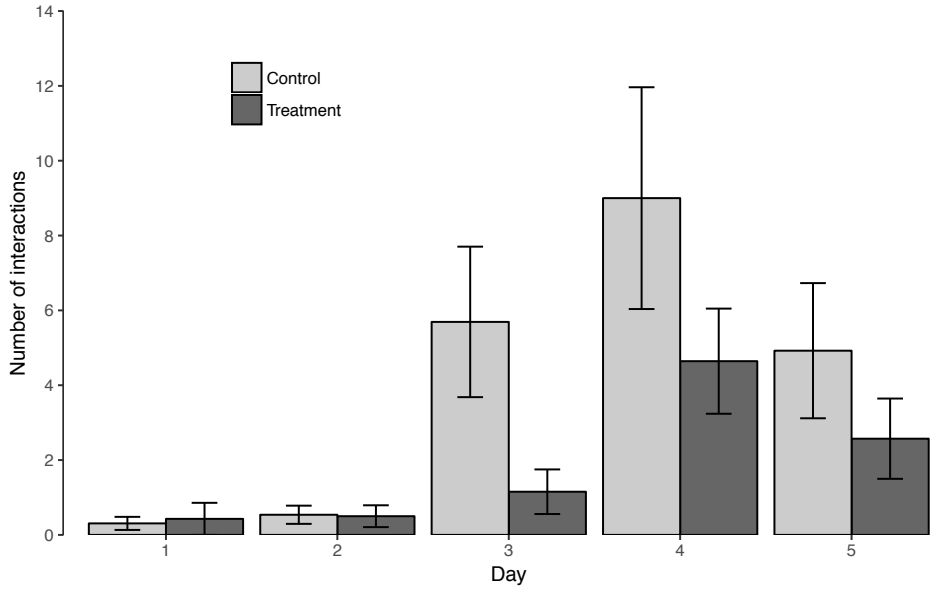

Figure 5-4. Agonistic interaction activity peaks on day 4 after microcolony establishment. Bar plot of the mean number of interactions on each day of the experiment in control microcolonies (light grey) and treatment microcolonies (dark grey). Error bars show ±standard error. Despite the appearance of a trend towards a lower number of interactions in treatment colonies compared with controls from Day 3 onwards, none of these comparisons was significant; this is likely due to the resulting small sample size of microcolonies in which all 5 bees survived the full course of the experiment.

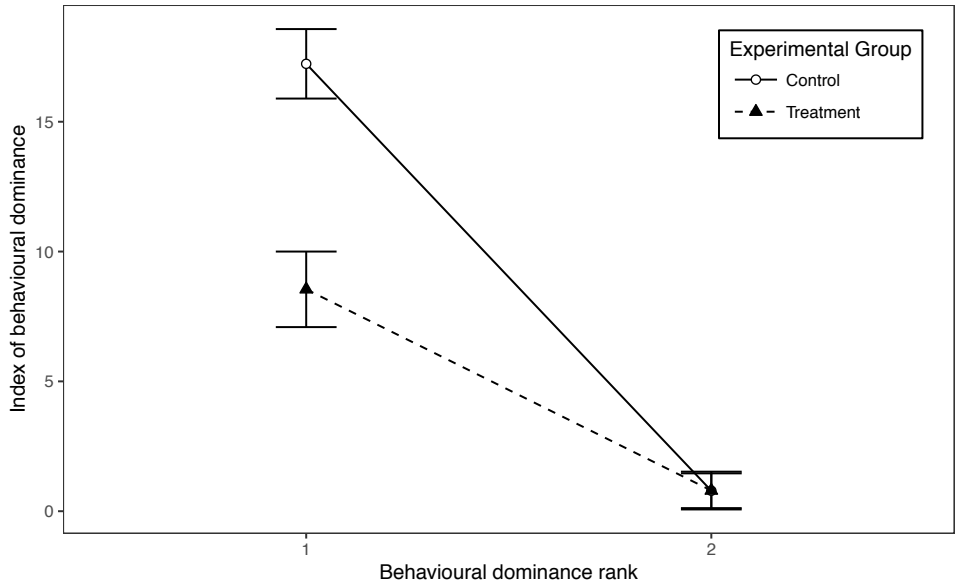

Figure 5-5. The majority of the decrease in the number of interactions in bumblebee microcolonies during pesticide exposure is observed in the alpha individual. Points show mean number of agonistic interactions initiated by the top ranked individual in each microcolony (Behavioural dominance rank 1) and all other bees within each microcolony (Behavioural dominance rank 2). Filled circles show control microcolonies, closed triangles show treatment microcolonies. Error bars show standard error. Ranks were determined based on the number of initiated agonistic interactions.

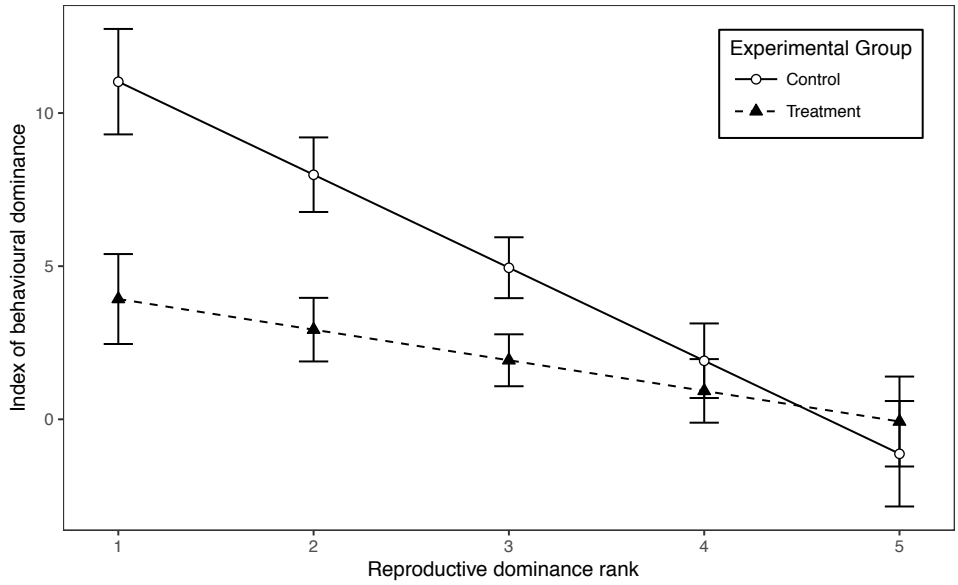

Figure 5-6. Bumblebees with larger ovaries tend to have higher indices of behavioural dominance, but no differential effect of pesticides with respect to reproductive dominance rank. Points show mean number of agonistic interactions initiated individuals according to position in the reproductive hierarchy. Filled circles show control microcolonies, closed triangles show treatment microcolonies. Error bars show standard error.

5.5 Discussion

Exposure to neonicotinoid pesticides is thought to be a principal driving force in the current declines in bumblebee populations seen across the industrialised world (Goulson et al., 2015). Although many neonicotinoid exposure experiments have described reductions in bumblebee colony growth (including number of adults, number of brood, colony weight, queen production) in queenright colonies (Feltham et al., 2014; Gill and Raine, 2014; Gill et al., 2012; Rundlöf et al., 2015; Whitehorn et al., 2012) and experimental microcolonies (Laycock et al., 2012; Laycock et al., 2014), the mechanism causing this effect is not fully understood. This study has taken the novel approach of recording behavioural dominance and reproductive dominance during neonicotinoid exposure. The results suggest that the behavioural effects of exposure are experienced primarily by dominant bees. This effect has the potential to affect brood production in queenless groups. Here I discuss the results in a wider context, critique the methodologies and outcomes, and identify future directions for research.

In line with previous work, average ovary development was not affected by pesticide exposure. Ovary development was also not affected any differently according to rank. This supports the suggestion by Laycock et al. (2012) that the process of oogenesis is relatively resilient to neonicotinoid toxicity. Neonicotinoids affect nerve cells in the bee brain (Palmer et al., 2013); therefore, we would not expect them to have a direct effect on ovary development. Nutrient limitation has often been cited as the cause of reduced brood production in pesticide-exposed colonies (Elston et al., 2013; Gill et al., 2012; Laycock et al., 2012). The current study found that treatment colonies consumed significantly less pollen than control colonies, while nectar was unaffected; this suggests that a 16.33% decrease in pollen consumption is not

significant enough to affect ovary development in groups of 5 workers. Other studies revealed that workers completely deprived of pollen show zero ovary development (Duchateau and Velthuis, 1989), while queens increase ovary development by 25% from stored energy alone, or by 50% from a nectar-only diet (Vogt et al., 1998). A possible explanation for the disparity between ovary development and reduced pollen consumption observed in this study could be that workers were able to invest in oogenesis from pollen consumed before the experiment. This would suggest that 5 days might not long enough to detect the effects of nutrient limitation on ovary development. Workers in the current study were less than 24 h old at the beginning of the experiment, which should have reduced the potential for feeding on pollen, but this remains a possibility nonetheless.

Pesticide exposure reduced agonistic interactions related to dominance in bumblebee colonies, which confirms Hypothesis 2a. Although movement behaviour was not measured in the current study, a reduction in movement speed, as shown in Chapter 3 provides a reasonable explanation for observed reduction in interactions. Reductively, this suggestion assumes that bees encounter one another randomly. This assumption may be valid as models of randomly interacting simulated bees can still form hierarchies based on winner/loser effects (Amsalem et al., 2013; Hogeweg and Hesper, 1983). Yet another possibility is that neonicotinoids affect the brain in a way that reduced the motivation of bees to engage in dominance interactions specifically, as opposed to the simple mechanics. Effects of neonicotinoids on aggressive behaviour have been shown in the argentine ant (*Linepithema humile*). Barbieri and Lester (2013) found that when *L. humile* colonies were exposed to sub-lethal doses of imidacloprid they became more aggressive during interactions with Southern ants (*Monomorium antarcticum*) and lost

more workers due to fights. The causes of this effect are not understood, but these findings highlight the complex and context-dependent ways in which this prevalent insect neurotoxin can affect social insect behaviour.

Although there was a reduction in the number of agonistic interactions, this did not seem to delay the peak in interactions when compared to the control. It was hypothesised (Hypothesis 2b) that a reduction in interactions would delay the establishment of the hierarchy, which was based on the assumption that the outcome of each interaction influences both bees involved according to winner/loser effects - a well established paradigm in studies of dominance hierarchies (Chase, 1982; Hogeweg and Hesper, 1983; Neumann et al., 2011). In this case, the day with the most interactions was used as a benchmark to time the development of the hierarchy. The results shown here, however, suggest that the timing is not affected by pesticide exposure.

Given that the total number of interactions was reduced by >50%, the stability in the timing of the peak of interactions is surprising if we assume each interaction is additive towards the establishment of a stable hierarchy. We could conclude that the addition of interactions is not the only factor affecting the dynamics of hierarchy formation. Additive interactions are thought to be sufficient to lead to differentiated ovary development in microcolonies (Amsalem et al., 2013), but ovary development is likely to cause subsequent changes to interaction behaviour. Within experimentally paired bees of *B. terrestris*, behaviourally dominant bees tend to develop their ovaries and cease producing a pheromone considered to represent a signal of sterility, while subordinate bees maintained production of this pheromone (Amsalem and Hefetz, 2010). This sterility signal seems to advertise subordination and to pacify dominant individuals, leading to reduced aggression (Amsalem and Hefetz, 2010; Amsalem et al., 2009). The

observed peak in aggression on day 4 may not have been delayed in the treatment group if unaffected ovary development altered the pheromone output of bees higher in the reproductive hierarchy. Aggression may be important for initiating initial differences in the dominance status of bumblebees, leading to subtle changes in ovary development that are amplified through positive feedback loops. Feedback loops are a key driver in self-organising systems (Bar-Yam, 1997b); therefore, the stability of the timing of hierarchy formation and unaffected ovary development could indicate the resilience of hierarchy formation to external disruption. There is also the possibility that the hierarchy formation may have been delayed by less than 24 h, but this experiment did not have resolution to test this.

The observed effect of exposure on agonistic interactions in this Chapter was primarily represented by a decrease in the number of interactions initiated by the bee in the alpha position of the behavioural dominance hierarchy. This finding is the opposite of Hypothesis 2c, which stated that the dominance behaviour of behaviourally subordinate bees would be more strongly affected. The rationale behind this hypothesis was the assumption that subordinate bees would have had behavioural dominance scores that: 1) could have been used to rank individuals on a 5-tier hierarchy, and 2) could have decreased. Instead, the high prevalence of ties for zero interactions resulted in 2-tier hierarchies and no detectable difference between subordinates in control and treatment groups. Although the data fail to support Hypothesis 2c, the possibility of low-dominance susceptibility (lower ranking bees more strongly affected) to neonicotinoid toxicity should not be ruled out until other (non-zero) behavioural responses are tested.

The idea of low-dominance behavioural susceptibility was also tested with respect to the *reproductive* dominance rank. The relationship between

reproductive dominance rank and dominance behaviour also suggested that the reproductive alpha was more strongly affected by exposure than bees with smaller ovaries. However, there was no statistical support for this observed trend.

With respect to queenright bumblebee colonies in the field, the observed effects on agonistic interactions and pollen consumption in this study could disrupt natural dominance hierarchies, but there is also evidence for resilience in ovary development and the hierarchy formation. Although neonicotinoid exposure reduces interaction rate, the self-organisation of dominance hierarchies was still functional. The presence of small initial inequalities in a system can grow exponentially if there are positive feedback loops that cause that past state of the system to affect the future state in a positive way (Bar-Yam, 1997b). Models have been used to simulate the situation where out of a group of initially identical bees, one will 'win' its first interaction by chance (Amsalem et al., 2013; Hogeweg and Hesper, 1983). In the model by Amsalem et al. (2013), winning increases the chance of winning in the future in a positive feedback loop. Also, the simulated bee with the most wins gains more ovary growth than the rest. These two principles are sufficient to generate linear reproductive dominance hierarchies. The initial wins by bees in this study could have been enough to trigger many more complex behavioural and physiological feedback loops that led to exponential changes in the system, even with fewer interactions. Thus, the relationship between the individual behaviour and group-level social structure may be more complex than the sum of agonistic interactions.

The prevailing hypothesis to explain reduced bumblebee colony productivity during neonicotinoid exposure is nutrient limitation (Gill and Raine, 2014; Gill et al., 2012; Laycock et al., 2012; Laycock et al., 2014). The

current study did not find evidence of this effect via an effect on ovary development, which suggests brood production in microcolonies is affected by some other mechanism. Ovary development in bumblebee queens, on the other hand, does seem to be affected by exposure. Baron et al. (2017a) showed that nest-founding bumblebee queens had reduced ovary development and reduced nectar consumption following exposure to thiamethoxam (5.32 ppb). Yet, reduced feeding did not completely explain the observed reduction in ovary development and the authors suggest some other component of neonicotinoid toxicity was generating this effect. Nevertheless, this effect reduced the chances that queens would initiate colonies by 26% (Baron et al., 2017b). In conclusion, the mechanism of the effect of neonicotinoid exposure on bumblebee colony productivity remains unresolved, but the effects on social behaviour and the implications of social resilience should be considered in future work.

The strength of the conclusions presented in this Chapter is unfortunately limited by the relatively low level of microcolony replication and by the low numbers of observed interactions between bees. The total number of microcolonies establish during the course of this experiment was 77, but only 27 were suitable to be used in the analysis. F. Wilkinson established and filmed 46 microcolonies between October 2017 and March 2018. C. Watrobska established and filmed the remaining 31 microcolonies between March 2018 and June 2018. Microcolonies were excluded from the analysis for technical issues during the experiment that would have confounded the results of the remaining dataset. Eight microcolonies were part of an early test of adding treatment to both nectar and pollen; however, this resulted in high mortality, killing nearly all of the bees. Three had one or more videos missing. This left the useable set of 27, of which only 19 could be used in the analysis that

matched behavioural scores and reproductive scores because of missing ovary measurements of dead individuals and the 4 microcolonies with zero interactions.

Increasing the number of replicates could have influenced the results of some of the trends in this study; for example: the difference in the daily peak (Figure 5-4), the relationship between the effect of behavioural rank and treatment on interactions (Figure 5-5), and the relationship between the effect of reproductive rank and treatment on interaction (Figure 5-6). However, the low number of interactions has been the greater problem in addressing the hypotheses set out in (Section 5.2). Low-ranking individuals were hypothesised to experience a stronger toxic effect of neonicotinoid exposure on their behaviour, while high-ranking individuals were expected to be relatively unaffected. The power of the experiment in testing this hypothesis was compromised by the low-ranking individuals most often engaged in zero interactions, even in control microcolonies. This was unexpected based on previous work that showed within 5-worker microcolonies (*B. terrestris*), the mean number of interactions initiated by the 4 subordinate workers was approximately 30 (over 120 minutes and across 12 microcolonies) (Amsalem and Hefetz, 2011: Figure 1, p. 4). In this study the mean number of interactions of the 4 subordinate workers was 0.79 (over 150 minutes, on average across 13 control microcolonies). Part of the reason for this difference was in the types of interactions recorded. Amsalem and Hefetz (2011) included "pushing" and "struggling" as directed agonistic interactions, but do not provide any further descriptions of their characteristics. For the current study, a "pushing-like" behaviour was sometimes observed and could be described as a slow version of "butting", i.e. the actor makes contact between its head and the recipient's body and continues to walk forcefully in

the direction of the recipient. However, this kind of pushing behaviour should not be included because its interpretation can be very subjective (S. Duckerin, personal observation) and it does not have a rigorous description that has been adopted across the literature. The interactions included in this study were strictly limited to clearly defined and easily recognised directed agonistic interactions that could be repeated by any other observer given the video data. The reasons for these discrepancies are unknown, but could be due to the use of a temperature controlled room at 30°C (Amsalem and Hefetz, 2011) versus a heated mat to maintain the nest box at 30°C (this study), the source and condition of the parent colonies, or the size of the nest boxes.

The microcolony system is advantageous because it can be more easily replicated than queenright colonies. The methodological decisions made in the design of the experiments in this Chapter were founded on evidence in the literature. However, the unexpectedly low number of interactions and the necessary exclusion of microcolonies that would otherwise have introduced errors, both limit the strength of the conclusions. Future studies aiming to record interactions should employ a number of additional measures to ensure sufficient interactions can be recorded for robust analysis. Improvements may include using more than five individuals per microcolony, which may result in a larger number of interactions per individual (Amsalem and Hefetz, 2011; Amsalem et al., 2013). However, larger microcolonies would greatly increase the experimental processing time in collecting sufficient callows >24 hours old on a given day (more parent colonies can help), marking individuals, extracting the behavioural data from the videos, and in dissecting the bees. Another improvement could be to increase the total observation time either by recording interactions for a longer period each day or by extending the

number of days in the experiment. With respect to the high prevalence of zeros interactions in this Chapter, even doubling the observation time would not have yielded a significant increase in recorded interactions among the subordinates. Moreover, extending the period of the experiment would present diminishing returns as the agonistic interactions peak on day 3-4 and thereafter decline (Amsalem et al., 2009).

In conclusion, the experiments in this chapter provide evidence that neonicotinoids disrupt the interactions involved in dominance hierarchy formation in microcolonies of *B. terrestris.* This effect has the potential to disrupt the social conditions required for egg-laying, but further research is required to test the relationship between dominance interactions in queenright colonies and if neonicotinoid exposure could impair brood production via a social mechanism. An understanding of this mechanism would be a significant contribution to the evidence base required for sustainable management and conservation of the ecosystem services provided by these insects.

Chapter 6

General Discussion

6.1 Overview

The future of the global agricultural system is going to be increasingly reliant on pesticide crop protection as food production demands will have to be met by substantially increasing the productivity of current land (United Nations et al., 2017; Zhang et al., 2006). When pesticides have the potential to affect the health of ecosystems, there is a trade-off between protecting current crop yields and protecting the ecosystem services that they rely on (Pimentel, 2009; Tison et al., 2016). Balancing trade-offs such as this is the fundamental challenge of sustainable food security and, if the balance is off, the outcome could affect the lives of many millions of people (FAO, 2017). For much of human history we have assumed that any impact we have on the Earth would be small-scale and short-lived. However, the global scientific community now recognises that the healthy functioning of many ecosystems are at risk from human activity (Barnosky et al., 2011; Waters et al., 2016). Scientific research now has a crucial part to play in understanding these risks and providing pragmatic solutions to overcome them. The current study contributes to this endeavour by highlighting the need for thorough pesticide risk assessment protocols that make connections across levels of biological

organisation to better understand the effects of these chemicals on the environment. By using bumblebee colony exposure to neonicotinoid pesticides as a specific case, this thesis illustrates how a complex systems approach to measuring human impact on natural systems can reveal impacts that may have been overlooked if system components were monitored in isolation.

6.2 Summary of Principal Findings

My thesis has contributed to pesticide risk assessment research by challenging the field to adopt a complexity approach to understanding the relationship between neonicotinoid exposure and bee colony decline. By employing high-throughput automated video tracking techniques, I was able to monitor every single individual within the bumblebee colony social system at the same time. This approach affords both fine-scale resolution and wide-scale coverage, and was used to describe the bumblebee system and its reaction to neonicotinoid exposure in new ways.

The technical aspects of this approach were introduced in Chapter 2. Automated methods are becoming increasingly popular tools in behavioural ecology as improvements in tracking technology and computational efficiency increase; this is especially so for video tracking (Dell et al., 2014). Automated video tracking can generate individual-level behavioural data at a degree of resolution and scale that is not possible through manual observation alone. However, despite its popularity, implementation of this approach is not yet a simple task. High quality (expensive) cameras, lights, and tags must be finely tuned to record image data that can be reliably interpreted by tracking algorithms. The result may contain unexpected errors, and so it is still important to perform regular manual checks of data quality and to correct sources of error when they appear. The computation time required to track

many hours of video can also cause severe delays to down-stream analyses. Chapter 2 demonstrates exactly how I overcame these challenges to produce a high-quality, high-resolution bumblebee colony video tracking dataset with more complete coverage than has been achieved previously. This vast dataset was then interrogated in Chapter 3 and Chapter 4.

Chapter 3 joins a wealth of literature illustrating the well-known importance of social context in modulating the behaviour of individual bumblebees (Amsalem and Hefetz, 2011; Amsalem et al., 2009; Leadbeater and Chittka, 2007); but my study went beyond previous pesticide-exposure work by showing, for the first time, that active foragerss and non-foraging workers vary in their behavioural susceptibility to, and recovery from, neonicotinoid exposure. Intranidal behaviour of active foragerss had not been examined in previous neonicotinoid exposure experiments: I showed that their movement speed was greatly reduced and their space use shifted significantly toward the nest centre. The movement speed of all other bees was also significantly reduced during pesticide exposure, and active foragers specifically did not recover movement speed when the pesticide was removed. These negative effects on intranidal forager locomotor behaviour were expected to have serious knock-on effects to foraging activity. At the individual-level, the time spent *inside* the nest in between foraging bouts was significantly longer during pesticide treatment, but the time spent *outside* the nest on foraging bouts was not affected. At the level of the superorganism, this did not result in a significant reduction in the number of foraging bouts, or a significant change in the number of foragers. However, the number of bouts per forager was significantly lower during exposure and previously active foragers seemed to reduce their foraging activity. Although these trends were not quite conclusive, they invoke the idea of a 'superorganismal response' to individual

behavioural impairment, i.e. the collective behaviour of many interacting individuals responding as one to overcome challenges in the environment. The wider relevance of these findings extends to providing recommendations for future methodologies employed in this field. Specifically, studies monitoring the behavioural responses of isolated individuals or of specific groups of individual (e.g. just foragers) are likely to have overlooked effects that are only apparent when the colony is considered as a whole. These findings highlight the importance of making connections levels of biological organisation, and that bee colonies should be considered as a superorganism, composed of many interacting individuals.

In Chapter 4, I built on the findings of Chapter 3 by examining how individual behavioural impairment scaled up to affect colony-level functioning. I achieved this by tracking changes in the structure and dynamics of colony social interaction networks before, during, and after pesticide exposure. Based on the findings of Chapter 3 and the literature, I predicted that the impairment of locomotor function during pesticide exposure would affect colony interaction patterns in ways that could disrupt fundamental colony processes, such as the organisation of work (Dornhaus and Chittka, 2001; Renner and Nieh, 2008; van Honk and Hogeweg, 1981). I presented two important findings. First, I described how bees in colonies exposed to pesticides interacted with a smaller diversity of other bees (i.e. the colony was less mixed), but still maintained normal rates of interactions with their remaining relations. Interaction rate remained stable during pesticide exposure despite a 25% reduction in movement speed (Chapter 3), implying that bees may have adjusted other aspects of their movement behaviour to compensate. The clustering of bees in smaller more central nest areas during pesticide exposure (described in Chapter 3) provides an explanation for the

stability of interaction rates: bees seem to be able to respond to interaction rate as a cue and to move toward areas that maintain interaction rate within certain threshold boundaries. This regulation of interaction rates has been described in ants (Gordon et al., 1993) and could constitute a simple and general behavioural algorithm at the individual level that helps to maintain stable interaction patterns at the superorganism level.

I then investigated the potential for the observed changes in social network structure to affect interaction patterns associated with forager recruitment. The interactions between all foragers and all non-foraging bees were not affected by exposure on a day-to-day basis, and neither were the dynamics of forager-related information flow. These results are exciting, as they suggest that there is network-level resilience to disruptions in the number of unique interaction partners per bee. It seems that part of this resilience is mediated via individual behavioural changes in space-use, as described in Chapter 3. The observed resilience in interaction patterns, despite individual behavioural impairment, is testament to the flexibility of individual behavioural responses that underpin the self-organisation of social processes in social insects.

In Chapter 5 I aimed to combine the approach of Chapter 3 (social modulation of behavioural response to pesticide) and Chapter 4 (effects on social interactions) with a measurable group-level outcome of social organisation: bumblebee dominance hierarchies. This study found that bees in microcolonies exposed to pesticides initiated fewer agonistic interactions overall. Upon inspection of the differences in this suppression of interactions across the hierarchy, it was apparent that subordinate bees (the four bottom ranked bees out of five) almost never initiated interactions in either the control group or the treatment group. Therefore, the main effect of pesticides

in reducing agonistic interaction was experienced by the single alpha individual (the top ranked out of five bees). This effect on dominance behaviour, plus the observed reduction of pollen consumption in the treatment group would have been expected to reduce ovary development (Amsalem et al., 2013; Duchateau and Velthuis, 1989; Hogeweg and Hesper, 1983). However, this study confirmed previous work demonstrating that this dose of the neonicotinoid imidacloprid does not affect average ovary development or reproductive skew (as measured by differences in ovary size within groups). The results of Chapter 5 make a case for considering the social effects on brood production, since ovary development does not seem to be directly affected. In this new scenario, a breakdown in dominance interactions could affect the social cues leading to egg-laying and cooperative brood care in more mature microcolonies. Further work is needed to clarify the ways in which neonicotinoid exposure affects the relationships between dominance interactions, pollen consumption, ovary development, egg-laying, and brood care.

6.2.1 The Superorganism Concept in Pesticide Risk Assessment

At the outset of this thesis, I proposed the idea of further integrating the concept of superorganismality into the way we approach pesticide risk assessment for social bees. Wheeler (1911) was the first to compare an ant colony to an organism, suggesting that individual ants can be equated in their role in the colony to the role of somatic cells in multicellular organisms. My thesis shows that in order to understand *why* colonies fail we must study the interactions between their component parts. In the same way as if we wanted to understand why neonicotinoid-exposed bees are less responsive to classical conditioning, we would have to study the interactions between nerve cells in

the brain (Hammer, 1997; Stanley et al., 2015a). Employing the concept of the bumblebee colony as a complex superorganism capable of displaying properties much more complex than any individual component has guided the narrative of this thesis. Overall, the results tell a story of individual impairment and superorganism resilience, suggesting the importance of considering effects across levels of biological organisation (including the level of the superorganism).

Chemically, neonicotinoids are synthetic alkaloids similar in structure to nicotine (Jeschke and Nauen, 2009). When insects are exposed to this water-soluble chemical (via topical application or dietary exposure), it is transported throughout the body to the central nervous system where it acts as a post-synaptic acetylcholine receptor agonist (Moffat et al., 2015; Moffat et al., 2016). This cellular mode of action causes nervous overstimulation, blockage of neurotransmission, and depolarisation of neurons in the nervous system (Palmer et al., 2013). The direct effect on the nervous system causes individual-level impairments to cognition and motor function (Chapter 3; Stanley et al., 2015). However, individual impairments and the capacity to recover from exposure are not experienced evenly across bumblebee societies (Chapter 3; Chapter 5). It appears as though these negative effects manifest particularly strongly in active foragers (Chapter 3; Gill and Raine, 2014; Gill et al., 2012), who may suffer as a result of high metabolic demands, navigating in a complex environment, or by ingesting relatively more toxin than other bees. The individual roles of non-foraging bees during pesticide exposure have not been thoroughly investigated, but behavioural impairments could disrupt intranidal activities such as brood care, nest defence or hierarchy formation (Chapter 3; Chapter 5).

Current evidence shows that neonicotinoids can affect the reproduction of the superorganism by limiting worker growth (analogous to the growth of somatic tissue) and the production of males and gynes (analogous to development of the gonads) (Gill et al., 2012; Laycock et al., 2012; Moffat et al., 2015; Rundlöf et al., 2015; Whitehorn et al., 2012; Woodcock et al., 2017). This effect on reproduction has been attributed to the relative inability of the superorganism to collect food during exposure, which manifests from a reduction in foraging efficiency at the individual level (Feltham et al., 2014; Gill and Raine, 2014; Gill et al., 2012; Stanley et al., 2016). The superorganism seems to retain the ability to respond to low food intake, as shown by the reallocation of more workers to foraging (Gill et al., 2012), resulting in each individual maintaining lower foraging activity (Chapter 3). The resilience of this superorganismal "behavioural" response to low food intake may be attributable to the resilience of fast information flow during exposure (Chapter 4). Perhaps the superorganism's "nervous system" (the network of information transmission between workers) continues to function despite individual behavioural impairment just as the nervous system of the individual bee continues to function despite individual neuronal impairment, albeit at a suboptimal level. Interestingly, this suggests that the superorganism exhibits resilience that is not based only on redundancy of individual components (Klein et al., 2017), but is more fundamentally based on the self-organisation of collective behaviour (Chapter 3; Chapter 4; Chapter 5). However, it seems that suboptimal system function can eventually affect reproduction in certain scenarios. We do not yet know the limits of social resilience in superorganisms, which system functions are most vulnerable to disturbance, or how to predict the success (or failure) of superorganisms faced with anthropogenic stressors. Yet, thanks to this study,

we do now know that answering these questions will require a complexity approach that considers the interactions that occur within systems and across levels of biological organisation.

6.3 Limitations and Further Work

6.3.1 The Dose Makes the Poison

The "field-realistic" dose used in laboratory studies is one of the most contentious subjects within neonicotinoid risk assessment research, and one that is very difficult to reconcile (see Carreck and Ratnieks, 2014). Undoubtedly, the most realistic exposure regime is attained by monitoring colonies in the field as they forage naturally among treated crops, but conducting controlled replicated landscape-scale experiments in agricultural land is a significant challenge. While laboratory studies are easier to conduct, retain more control, and can offer results of greater resolution, simulating a field realistic dose may not be straightforward. This is because the amount of active substance in the bee brain arriving via oral exposure is determined by the *concentration* of neonicotinoid in treated food, and by factors affecting the *dose* such as, the type of treated food, the option to choose between treated and untreated food, and the duration of exposure (Carreck and Ratnieks, 2014).

Field-realistic concentrations of neonicotinoids are measured either by taking pollen and nectar samples from seed-treated bee-attractive crops, or from the pollen and nectar collected by bees. Concentrations from these samples show significant variation across countries, chemicals and crops (Bonmatin et al., 2014; Cutler and Scott-Dupree, 2014; Schmuck, 1999; Scott-Dupree et al., 2001; Woodcock et al., 2018). However, the literature generally considers concentrations ≤10 ppb to be field-realistic (Gill and Raine, 2014;

Gill et al., 2012; Henry et al., 2012; Whitehorn et al., 2012), while some suggest the upper end of this range represents only a worst-case scenario exposure (Bonmatin et al., 2014; Carreck and Ratnieks, 2014; EFSA, 2012; Gels et al., 2002). Nevertheless, simple measurements of concentration can be confounded by other factors in laboratory oral exposure experiments.

The duration of exposure is a key variable that should, where possible, either match natural exposure regimes or be used to track effects over time. A 'natural' exposure time is sometimes based on the mass-flowering of oilseed rape crops (often treated with neonicotinoids), which can last approximately 3 weeks and make up a significant proportion of the diet of foraging bees during this time (Heinrich, 1979; McCartney and Lacey, 1991). Replicating this kind of 'pulsed' exposure in the laboratory is important to understand how bees might respond when their primary source of food is from a single, ephemeral treated crop. This assumes: 1) that bees do not adjust their foraging choices in response to pesticide contamination, and 2) that bees will have a 'safe' untreated food source after a natural pulse, neither of which may be true. Bees actually show a preference for neonicotinoid-treated nectar in laboratory experiments (Arce et al., 2018; Kessler et al., 2015) and neonicotinoids appear to be commonly found in wildflowers (Botías et al., 2015; David et al., 2016), which could affect foraging choices and prolong exposure. In conclusion, replicating the exposure profile of free-foraging bees in the laboratory is complicated and the best approach is up for debate. The concentration of imidacloprid used here (10 ppb) is at the upper end of concentrations detected in crop nectar and may be considered unrealistic by some, but pollen was provided untreated and exposure was only for one week; therefore, this represents a plausible exposure challenge that could approximate a natural pulsed exposure. When this pulse of exposure was

incorporated into the baseline-experiment-reversal design of the experiments in Chapter 3 and Chapter 4 it provided a complete picture of the changes in bee and colony behaviour that can be compared across studies.

My thesis shows that the *effects* of neonicotinoids are not equal across the colony (Chapter 3; Chapter 5). These findings should challenge the widely held assumption in the literature that *exposure* is also equal within a colony. Throughout my thesis I have adopted the assumption that inter-individual variation in susceptibility to neonicotinoid exposure occurs as a result of physiological differences driven by the conditions of performing different tasks, e.g. reduced immunity in *Apis mellifera* foragers (Amdam et al., 2005). However, an alternative is that individuals vary in the dose that they receive, which, to my knowledge, has not been tested in the context of bees and neonicotinoids. The behaviour of active foragers, for example, was strongly affected, and their locomotor function did not recover (Chapter 3). The possibility that foragers are more likely to experience the negative effects of toxicity because they were subjected to a higher dose of pesticide while transporting loads of contaminated nectar has not yet been investigated. Furthermore, we do not know how evenly neonicotinoids are spread throughout the food stores of the colony and among the colony members, and how long they persist in food stores. Answers to these questions could help us to understand the vulnerabilities of certain groups of individuals, which could aid in producing accurate mechanistic models of exposure to make more powerful predictions of colony responses (Sponsler and Johnson, 2017). This information could also reveal facets of social organisation that could serve to protect the colony, such as the supposed existence of 'silos' in ant colonies. These 'silos' individuals store large amounts of food, apparently serving to

check whether it is safe before passing it on to the rest of the colony (Sendova-Franks et al., 2010).

The capacity for bees to recover from exposure to neonicotinoids is another important consideration when comparing the doses and effects used in different studies. My thesis and several others studies consider the 'pulsed' regime as an opportunity to track how bees respond during exposure and how they recover after exposure (Cresswell et al., 2013; Laycock and Cresswell, 2013). However, there is a lack of continuity in the literature about how we refer to the exposure and post-exposure stages of an experiment and address this could clarify the relationship between certain results. For example Whitehorn et al. (2012) exposed bumblebee colonies to dietary neonicotinoids in the laboratory for 2 weeks and then moved the colonies to the field where they were free to forage. This study tracked weight gain and gyne production throughout and found no effects during the 2-week exposure phase, but described significantly less weight gain in treated colonies during the 4-week post-exposure phase and an 85% reduction in the final gyne production. As another example, Chapter 3 and Chapter 4 tracked behavioural effects during a 1-week exposure phase and a 1-week post-exposure phase. The results showed significantly reduced mixing of bees inside the nest during exposure, but that this behavioural effect was almost complete reversed post-exposure. Putting these together we might conclude that short-term individual behaviour and social cohesion are resilient to exposure, while long-term brood production is much more sensitive to disruptions. These examples highlight the many scales across which neonicotinoids can affect bee colonies and the importance of improving the way we make comparisons across studies.

6.3.2 Automation in the Field

If I were to be given another 3 years to extend this project I would aim to scale up the fine-scale, colony-wide behavioural monitoring used in this thesis to semi-field and field exposure experiments. This may be the most powerful approach to addressing the controversy surrounding the harmful effects of neonicotinoids on bees because it addresses at least two key issues. The first is the "field-realistic" dose, which is only provided with certainty in field exposure experiments. The second is the issue that some studies find harmful effects while others do not. The 'superorganism' approach of tracking all individuals as constituent parts of a complex system could resolve the differences between contradicting studies by describing effects across levels of biological organisation. For example, if Chapter 3 had recorded only the number of foraging bouts, there would have been no detectable effect of exposure on colony-wide foraging effort, perhaps leading to the conclusion that the colony was not affected. However, individual-level measurements revealed both active foragers and non-foraging workers were individually affected in ways that did not manifest at the superorganism level. Equally, if Chapter 4 had not considered bumblebee social interactions as a temporal network, the effects on individual locomotor behaviour could have been used to overstate the potential for negative effects on colony functions such as responsiveness to new information. Yet, despite individual behavioural impairment, information flow within the superorganism appears to be maintained. These findings suggest that previous studies that have described "no-effect" at the colony level may fail to record the fact that the colonies were operating under high stress and may have been at the edge of their limit. In the future it will be important to measure the limits of social resilience to help us reconcile contradicting studies.

In an ideal world, this behavioural monitoring set-up would be applied to track the behaviour of colonies in the field, and would address the issues of dose and mixed effects all in one experiment. In reality, there are many challenges that must be overcome to achieve this, but many lessons can be learnt from this study.

6.3.3 Technological and Computational Challenges

During this study, the volume and resolution of video/image data collected (8TB of raw image data) combined with the relatively slow speed of the BEEtag tracking software resulted in a very long wait to produce the tracking data. This could constitute a serious limiting factor in scaling up this experiment to more colonies. The first potential improvement in this wait time could come from a reduction in the volume of image data. Future studies should carefully consider the resolution required to generate data appropriate to the question be answered. Reducing the frame rate and/or the total recording time are two simple solutions to this issue. Next, the computation efficiency of the BEEtag software could be improved. This is a likely possibility given the open-source availability of the software. There are many other approaches and solutions for automated behavioural monitoring, and there is much active development in this area (Gernat et al., 2018; Mersch et al., 2013; Noldus et al., 2001; Pérez-Escudero et al., 2014; Rodriguez et al., 2018; Yamanaka and Takeuchi, 2018). Moving forward, there should be increased access to open source software and more collaboration between biologists and computer scientists to make automated techniques more widely accessible and to avoid "reinventing the wheel".

In a study published this year, Crall et al. (2018), developers of the BEEtag system, demonstrated the application of this system in the field.

They subjected colonies to the removal of foragers and showed that the individuals that replaced them tended to be associated with nectar pots. Importantly, they demonstrated that is it possible to apply this technique in the field on a large scale. However, there was a trade-off in the extent of colony-wide coverage. Once the colonies were out the field they were not inspected internally until the end of experiment, which means newly emerged bees were not marked, lost tags were not replaced and tags obstructed with wax were not cleaned. These three processes were extremely time consuming during the implementation of the current study, but they ensured near-complete coverage of colonies. Future studies may need to consider the trade-off between full coverage and field-realism.

6.3.4 The Potential Effects of Neonicotinoids on Nest Defence

The effect of neonicotinoid exposure on bumblebee colony nest defence could be a another natural follow-up laboratory experiment to this study. Bumblebee colonies emit a conspicuous buzzing sound when disturbed by either substrate vibrations or CO_2 (an indicator of mammalian breath) (Kirchner and Röschard, 1999). Nest defence is also marked by several different defensive behaviours, which include leaving the nest in attack, running around the inside the nest ('patrolling'), or standing stationary with antennae raised ('perching') (see Jandt et al., 2012). Some colonies exposed to 10 ppb imidacloprid in this study were completely unresponsive to substrate vibrations in terms of buzzing and patrolling (S. Duckerin, personal observation). Imidacloprid-exposed colonies have also been overrun with wasps in a field experiment (Moffat et al., 2015). Interestingly, a recent study has also described negative effects of exposure on the process of buzz pollination (Whitehorn et al., 2017). Imidacloprid exposure could reduce the

defensive response of bumblebee colonies, but this hypothesis has not yet been tested. This could represent a serious behavioural impairment for colonies faced with predation by mammals that would normally be deterred by the defensive response (Kirchner and Röschard, 1999). A microphone could be used to the record the colony-level average buzzing sounds of neonicotinoid-exposed colonies as a simple test of this hypothesis. Automated tracking could also be used to record the collective movement behaviour of individuals inside the colony in response to disturbance. Bumblebees also show individual variation in the propensity to display certain defensive behaviours (Jandt et al., 2009); therefore, as in Chapter 3, there may differential behavioural effects on individual defence responses.

6.4 Concluding Remarks

Pesticide risk assessment research has a crucial part to play in ensuring a sustainable future for food security. We cannot feed the world without pollinating bees, and we also cannot feed the world without pesticides. Balancing this trade-off requires a thorough understanding of the costs and benefits pesticide use, versus the potential for environmental harm. I suggest that pesticide exposure research should embrace complexity theory to help describe and understand the complex systems and their interactions at play in this contemporary issue. I have shown how this approach can deepen our understanding of how agricultural chemicals interact with natural systems.

The familiar hum of the buzzing bumblebee is one of the most recognisable sounds of summer in the British countryside. As such, I was shocked to find that after only a couple days feeding on contaminated nectar, my laboratory colonies fell totally silent. They were noticeably lethargic. This personal experience, combined with the wealth of evidence described in this

thesis, has convinced me that neonicotinoids pose a serious exposure risk to bumblebees. The use of neonicotinoids should be very carefully regulated, if they are used at all. As of April 2018, three major neonicotinoid pesticides were banned from agricultural use throughout the European Union. I think this is an appropriately cautious decision given the risk and I think more countries should carefully consider the use of these pesticides in agricultural land. However, if current prophylactic pesticide application practices continue, banning neonicotinoids will turn farmers to alternatives, which could cause just as much harm (Siviter et al., 2018). I strongly believe that pesticides should only be used according to the principals of Integrated Pest Management, an ecosystem approach to crop production defined by the UN's Food and Agriculture Organization as, "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO, 2018).

In conclusion, although I have described some aspects of colony-level resilience to neonicotinoid exposure, this does not mean that bees are immune; there will be a limit to this resilience. We must identify the limits to social resilience in bumblebees and ensure that wild colonies in agricultural land are never pushed past it if we are to conserve these amazing insects and attain food security.

Appendices

Appendix 1: The relationship between reproductive dominance rank and dominance behaviour with and without microcolonies containing >5 individuals.

Continued on next page.

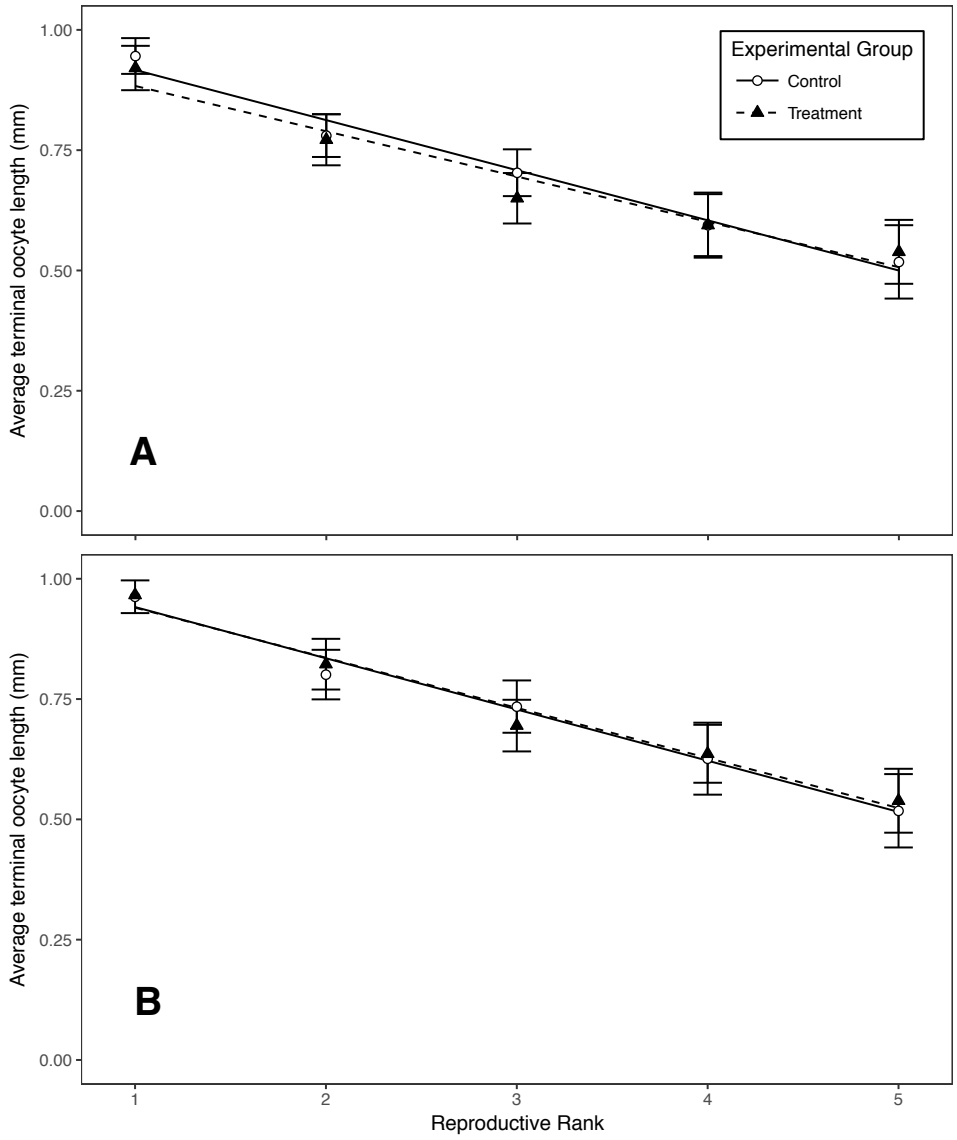

Figure 6-1. Linear reproductive dominance hierarchy not affected by pesticide exposure. Bees in each microcolony were assigned a rank according to their ovary development (average terminal oocyte length). Mean ovary development is shown for each rank in control microcolonies (open circles), and treatment microcolonies (closed triangles). Error bars show ± standard error. Lines show linear regression results; solid line = control, dashed line = treatment. Panel A shows results of all microcolonies, including those with missing ovary measurements due to early mortality during the experiment. Panel B shows the stronger relationship when microcolonies that had missing data were excluded.

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